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## REMARKS

Rejection of Claims 1-4, 11 and 12 Under 35 U.S.C. 102(b)

Claims 1-4, 11 and 12 are rejected by the Examiner under 35 U.S.C. 102(b), for the reasons set forth in paragraphs 9-10 of the Office Action. This rejection is respectfully traversed. Reconsideration and withdrawal thereof are requested.

Applicants hereby clarify the comments in the last paragraph on page 14 of the Remarks filed on July 1, 2004. The comments in the last paragraph on page 14 of the Remarks filed on July 1, 2004 should be replaced with the following corrected comments:

> However, as described in the specification, the present inventors' view was that other than the enzymatic activity reported by Gately et al., another unknown enzyme might possibly be involved in the production of angiostatin. As to the enzymatic activity reported by Gately et al, the enzyme per se had not yet been isolated and identified at the time but later purified to reveal that a serine protease called plasmin and free cysteine donors were responsible for the enzymatic activity (Gately et al., Proc. Natl. Acad. Sci. USA, Vol. 94, pp.10868-10872 (1997); see attached copy).

In addition, Applicants previously indicated that a Rule 132 Declaration will follow. For instance, see the comments in the second paragraph on page 14 of the Remarks filed on July 1, 2004. Attached please find an executed Rule 132 Declaration which should be made of record in the present application. The Declaration explains why the cited reference neither anticipates nor suggests the present invention.

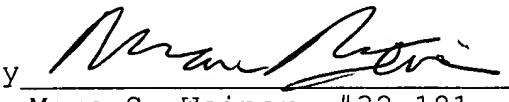
Accordingly, this prior art rejection should be withdrawn in view of the remarks hereinabove and in view of the discussion in the attached Declaration.

Should there be any outstanding matters that need to be resolved in the present application, the Examiner is respectfully requested to contact Marc S. Weiner (Reg. No. 32,181) at the telephone number of the undersigned below, to conduct an interview in an effort to expedite prosecution in connection with the present application.

If necessary, the Commissioner is hereby authorized in this, concurrent, and future replies, to charge payment or credit any overpayment to Deposit Account No. 02-2448 for any additional fees required under 37 C.F.R. §§ 1.16 or 1.17; particularly, extension of time fees.

Respectfully submitted,

BIRCH, STEWART, KOLASCH & BIRCH, LLP

By 

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MSW/sh
0020-4841P

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of
Toru KAWAI et al.
Serial No. 09/806,568 Group Art Unit: 1642
Filed: July 30, 2001 Examiner: A. Harris
For: ENZYME PRODUCING PLASMA PROTEIN FRAGMENT HAVING INHIBITORY ACTIVITY TO METASTASIS AND...

DECLARATION

Honorable Commissioner of Patents and Trademarks
Washington, D.C. 20231
U.S.A.

Sir:

I, Wataru MORIKAWA, a citizen of Japan residing 14-29, Kusunoki 7-chome, Kumamoto-shi, Kumamoto-ken, Japan, declare as follows:

1. I was graduated from Shimane University, Faculty of Agriculture, Department of Agricultural chemistry in 1983, finished the graduate school (master course) in said University in 1985, and was conferred a doctorate of medicine from Kyushu University, a postgraduate course, medical department through the examination of a thesis in 2003.

2. Since 1985 up till the present, I have been in the employ of Juridical Foundation The Chemo-Sero-Therapeutic Research Institute. Since 1999 up till 2002, I have been prosecuting the study of angiogenesis inhibitor at Kyushu University, Faculty of Medicine, Chair of First Biochemistry.

From April 1985 up till 1987, I had been engaged in the research work with respect to development of blood coagulation factor, from 1987 up till 1995, to development of blood coagulation factor and investigation of useful factors in blood (study on relationship between lipoproteins such as HDL or Lp(a) and arteriosclerosis), from 1995 up till 1999, to investigation of angiogenesis inhibitor (angiostatin), and from 2002 up till the present, to development of recombinant blood coagulation factor at said Foundation.

3. For the past years, I had made several reports as listed below:

1) Acetylated low density lipoprotein reduces its ligand activity for the scavenger receptor after interaction with reconstituted high density lipoprotein, J. Biol. Chem., 1994 Feb. 18: 269(7): 5264-5269

2) Comparison of monoclonal and polyclonal enzyme-linked immunoabsorbent (ELISA) assays for serum Lp(a) and differences in reactivities to Lp(a) phenotypes, J. Clin. Lab. Anal., 1995: 9(3): 173-177

3) Measurement of Lp(a) with a two-step monoclonal competitive sandwich ELISA method, Clin. Biochem., 1995 June: 28(3): 269-275

4) Intravenous injection of rabbit apolipoprotein A-I inhibits the progression of atherosclerosis in cholesterol-fed rabbits, Arterioscler. Thromb. Vasc. Biol., 1995 Nov.: 15 (11): 1882-1888

5) Reconstituted high density lipoprotein reduces the capacity of oxidatively modified low density lipoprotein to accumulate cholesterol esters in mouse peritoneal macrophages, Atherosclerosis, 1996 Jan. 26: 119(2): 191-202

6) Lipoprotein (a) induces cell growth in rat peritoneal macrophages through inhibition of transforming growth factor-beta activation, Atherosclerosis, 1996 Aug. 23: 125(1): 15-26

7) The activity of soluble VCAM-1 in angiogenesis stimulated by IL-4 and IL-13, J. Immunol., 2000 Sep. 1: 165(5): 2818-2823

8) Angiostatin generation by cathepsin D secreted by human prostate carcinoma cells, J. Biol. Chem., 2000 Dec. 8: 275(49): 38912-38920

9) New functional aspects of cathepsin D and cathepsin E, Mol. Cells. 2000 Dec. 31: 10(6): 601-611

10) The accumulation of angiostatin-like fragments in human prostate carcinoma, Clin. Cancer Res., 2001 Sep.: 7(9): 2750-2756

11) Downregulation of Cap43 gene by von Hippel-Lindau tumor suppressor protein in human renal cancer cells, Int. J. Cancer, 2003 Jul. 20: 105(6): 803-810

12) Cellular distribution of NDRG1 protein in the rat kidney and brain during normal postnatal development, J. Histochem. Cytochem., 2003 Nov.: 51(11): 1515-1525

4. I am a member of The Japanese Society for

Pediatrics.

5. I am one of the inventors in the instant U.S. application and familiar with the subject matter thereof.

6. I have read Gately et al., Cancer Research 56: 4887-4890, 1996, and am familiar with the subject matter thereof.

7. It is my opinion based on my knowledge and experience in this field that the enzyme as well as a product therefrom of plasminogen according to the present invention are distinct from those of Gately et al.

**(1) General description of an enzyme**

An enzyme is a protein that acts as a biocatalyst to catalyze various chemical reactions within the living body with a strict substrate specificity acting only on a specific substrate, i.e. cleaving a specific amino acid sequence of a specific protein in case of a protease, a kind of an enzyme that cleaves an amino acid sequence of a protein as a substrate.

A protease, including those with a variety of activities such as e.g. one that merely digests a protein and one that activates or inactivates other proteases, may be classified into a number of groups based on an amino acid residue at an active center, including a serine protease, a cysteine protease, an aspartate protease, etc.

Most importantly, each protease has a specific pH range at which an enzymatic activity may be exerted, said pH range being dependent upon an electric charge of an amino acid residue residing at an active center. Thus, a protease may only be active at a specific pH range but not at one beyond said pH range.

Specifically, a serine protease may be active at neutral pH but not at acidic pH whereas an aspartate protease may be active at acidic pH but not at neutral pH (see e.g. Michael J. North, Microbiological Reviews, Sept., pp.308-340 (1982), "Comparative Biochemistry of the Proteinases of Eucaryotic Microorganisms"; a copy attached hereto).

**(2) Summary of Gately et al.**

Lung and liver metastasis of PC-3 human prostate

carcinoma cells in athymic mice remain at the microscopic stage whereas the primary tumor increases 4-fold in size. These data suggest that PC-3 cells express a factor that suppresses the growth of metastatic tumor cells (Introduction at p.4887).

On the assumption that the antimetastatic factor is an angiogenesis inhibitor, angiostatin, Gately et al. reacted plasminogen with culture supernatant of PC-3 cells to thereby obtain angiostatin-like fragments (Fig. 1 at p.4888), revealing that an enzymatic activity able to produce angiostatin-like fragments is present in culture supernatant of PC-3 cells.

Said enzymatic activity is estimated as a serine protease in consequence of proteinase inhibitor analysis (Table 1 at p.4889) wherein several serine proteinase inhibitors exhibited inhibitory activity while aspartic proteinase inhibitor, pepstatin, showed no inhibitory activity.

The enzymatic activity of Gately et al. produced angiostatin-like molecule with a molecular weight of approximately 50 kD ("Angiostatin Generation by Prostate Cancer Cells" at p.4888, second col.). The angiostatin-like molecule of Gately et al. was later demonstrated to comprise Kringle 1 to Kringle 4 and part of Kringle 5 (P. Stathakis et al., The Journal of Biological Chemistry, Vol.274, No.19, p.8910-8916, 1999; a copy attached hereto). It is also demonstrated that said angiostatin-like molecule has an activity to inhibit angiogenesis likewise angiostatin both in vitro (Fig. 2 at p.4889) and in vivo (Fig. 3 at p.4890).

**(3) Comparison of the enzyme of the present invention with the enzymatic activity of Gately et al.**

It is true that the present invention has been completed starting from study of Gately et al. In fact, the present inventors have recognized the presence of an angiostatin-producing activity in PC-3 culture supernatant as reported by Gately et al. It is also true that the present inventors have tried to purify an enzyme responsible for said enzymatic activity earlier than Gately et al. would have tried.

However, as described in the specification, the

present inventors' view was that other than the enzymatic activity reported by Gately et al., another unknown enzyme might possibly be involved in production of angiostatin. As to the enzymatic activity reported by Gately et al., the enzyme per se had not yet been isolated and identified at the time but later purified to reveal that a serine protease called plasmin and free cysteine donors were responsible for the enzymatic activity (Gately et al., Proc. Natl. Acad. Sci. USA, Vol. 94, pp.10868-10872 (1997); a copy attached hereto).

Contrary to disclosure of Gately et al., the present inventors have successfully found, in addition to an enzyme that fragments plasminogen at neutral pH, that an enzyme activity that can specifically cleave a restricted site of plasminogen under a lower pH condition was present in PC-3 culture supernatant. This enzymatic activity has not yet been reported previously and is utterly different from the enzymatic activity of Gately et al.

The most important aspect in considering distinction between the enzymatic activity of Gately et al. and the enzyme of the present invention is a pH range at which an enzymatic activity may be exerted.

Fig. 1 of the instant application indicates that fragmentation patterns of plasminogen by PC-3 culture supernatant, which culture supernatant is identical to that used by Gately et al. with respect to the source of the cells, culture medium and culture conditions, vary with a pH range at which PC-3 culture supernatant is placed. Specifically, at around neutral pH where a serine protease is active, appearance of angiostatin-like protein as reported by Gately et al. was detected at a corresponding molecular weight (Namely, the present inventors as well have detected in fact the enzymatic activity as reported by Gately et al.). On the other hand, when pH is shifted to an acidic range, the activity to produce said angiostatin-like protein disappears while instead a distinct angiostatin-like protein thought to be a cleaved product by a distinct acidic protease is detected at a different molecular weight from that of Gately et al. This finding at an acidic pH range for the enzymatic activity and the cleaved product therefrom is just the present invention, i.e. an aspartate protease according to the present

invention (PACE4) and an angiostatin-like protein obtained from plasminogen with said enzyme.

The present inventors have focused on said enzymatic activity that is only active at an acidic pH range to cleave plasminogen to produce angiostatin-like protein so that an enzyme responsible for said enzymatic activity may be purified and identified, which enzyme however is distinct from the enzymatic activity of Gately et al.

Although Gately et al. do not expressly mention at which pH the enzymatic activity is detected, it should be noted that the enzymatic activity of Gately et al. is inhibited by a serine proteinase inhibitor but not by an aspartic proteinase inhibitor as shown in Table 1, demonstrating that said enzymatic activity is of a serine protease. In this regard, it should also be noted that a serine protease works only at a neutral pH range but not at an acidic pH range, e.g. pH 3 or 4, due to the electric charge of serine at the active center. Table 1 of Gately et al. indicates that only serine proteinase inhibitors blocked angiostatin generation but none of other classes of proteinase inhibitors including an aspartate proteinase inhibitor such as pepstatin were effective (bridging paragraph between pages 4888 and 4889). In other words, Gately et al. failed to detect the presence of any enzymatic activity that could be blocked by an aspartate proteinase inhibitor, i.e. the presence of an aspartate protease like PACE4 according to the present invention.

Contrary to Gately et al., the enzyme of the present invention is an aspartate protease as being inhibited by an aspartic protease inhibitor and works only at an acidic pH range (page 9, lines 19-21 of the specification).

With the knowledge that the enzymatic activity of Gately et al. is of a serine protease and that a serine protease is only active at a neutral pH range, one of ordinary skill in the art would not have been motivated to investigate whether any acidic protease like the enzyme of the present invention is present in PC-3 culture supernatant. The present inventors, however, previously recognized possibility of presence of such an enzyme with an angiostatin-like activity that acts at an acidic pH range as described in the specification, thus leading to

accomplishment of the present invention.

In summary, the enzyme of the present invention is an aspartate protease acting at an acidic pH range whereas the enzymatic activity of Gately et al. is a serine protease acting at a neutral pH range with evidence that the former is inhibited by an aspartate protease inhibitor while the latter by a serine protease inhibitor. It should be noted that an aspartate protease and a serine protease are quite distinct enzymes from each other as a matter of course.

As a consequence of difference between the enzyme of the present invention and the enzymatic activity of Gately et al., they recognize and cleave plasminogen at different sites of an amino acid sequence thereof to produce thereby different cleaved products, in this case angiostatin-like proteins. More specifically, the enzyme of the present invention produces from plasminogen angiostatin-like molecule with a molecular weight of 40 or 43 kDa comprising Kringle 1 to Kringle 4 whereas the enzymatic activity of Gately et al. produces angiostatin-like molecule with a molecular weight of about 50 kDa comprising Kringle 1 to Kringle 4 and part of Kringle 5. These two products are distinct from each other but happen to have the similar activity, i.e. an activity to inhibit angiogenesis.

8. Viewing the above matters, it is my opinion that the enzyme and a product therefrom of plasminogen of the present invention is distinct from the enzymatic activity revealed in PC-3 culture supernatant and a product therefrom by Gately et al.

The undersigned declares further that all statements made herein of his own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the above-mentioned application or any patent issuing thereon.

This 2nd day of July, 2004.

Wataru Morikawa

Wataru MORIKAWA

MICROBIOLOGICAL REVIEWS, Sept. 1982, p. 308–340
0146-0749/82/030308-33$02.00/0

Vol. 46, No. 3

# Comparative Biochemistry of the Proteinases of Eucaryotic Microorganisms

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## INTRODUCTION

Traditionally the proteinases have been regarded as degradative enzymes which are capable of cleaving proteins into small peptides and amino acids and whose role it is to digest nutrient protein or to participate in the turnover of cellular protein. Indeed, this is true of the best characterized of the proteinases, such as the mammalian digestive enzymes trypsin, chymotrypsin, and pepsin and the lysosomal enzymes cathepsin B and cathepsin D. More recently, however, it has also been demonstrated that limited proteolysis has a key role in a wide range of cellular processes (see references 117 and 118). The ability of proteolytic enzymes to carry out selective modification of proteins by limited cleavage, as in the activation of hormones, for example, means that some proteinases have a regulatory function. This has added considerable interest to an already important group of enzymes. As a result of the recognition of more specific proteolytic processes and the use of more selective substrates, an increasing number of proteinases are being detected in all types of organisms. This review is concerned with the proteinases of one group, the eucaryotic microorganisms.

There are a number of reasons why the proteinases of these organisms are of particular interest. A number of species, for example,

*Saccharomyces cerevisiae*, *Neurospora crassa*, *Aspergillus nidulans*, *Physarum polycephalum*, *Dictyostelium discoideum*, and *Tetrahymena pyriformis*, are widely used for analyzing the molecular basis of a range of physiological and development processes. A study of the proteolytic enzymes of these species contributes to our understanding of the role played by proteinases and peptidases in many cellular functions involving proteolysis, including intracellular protein turnover, digestion, protein translocation, sporulation, and germination. Knowledge of the properties of these enzymes may also aid the development of methods which prevent artifacts caused by proteolysis during preparation of cellular materials (263). Many species of eucaryotic microorganisms are pathogenic to humans, livestock, or crops. Since proteolysis may play a number of roles in pathogenesis, for example, in the penetration of the host organism, in countering host defense mechanisms, and in nutrition during infection, an analysis of the proteolytic enzymes may prove invaluable to an understanding of pathogenesis and might suggest means of controlling the pathogens.

Furthermore, a study of proteolytic enzymes is merited because of their importance as reagents in laboratory, clinical, and industrial processes. Proteinases from a number of sources, both microbial and nonmicrobial, are in widespread use in the food industry (baking, brewing, cheese manufacturing, meat tenderizing), in the tanning industry, and in the manufacture of biological detergents (9). The use of fungal proteinases is of particular importance in the food industry. Many traditional food processes, especially the preparation of Oriental fermented foods, are also dependent on proteolytic microorganisms.

Thus, there is an increasing interest in the proteinases and peptidases of the eucaryotic microorganisms. A survey of the literature since 1973 indicates that proteolytic activity has been reported in species representing over 150 genera of fungi, protozoa, and slime molds. For some genera, for example, *Aspergillus* and *Penicillium*, many different species are of interest. It is not possible to provide a complete account of all the enzymes in all species in this review. Consequently, the contents are restricted to a discussion of the proteinases (the endopeptidases), although, since a complete understanding of proteolysis must take into account the role of exopeptidases (aminopeptidases, carboxypeptidases, and dipeptidases), these are not ignored completely. Emphasis is placed on the areas of most recent interest and those which have not previously been reviewed. This applies in particular to the proteinases of the protozoa and slime molds. For more detailed descriptions of other areas, the reader should consult the various reviews referred to in the text.

## PROTEINASE PROPERTIES

From an analysis of their in vitro properties, proteinases may be classified in a number of ways, for example, on the basis of the pH range over which they are active (acid, neutral, or alkaline), on the basis of their ability to hydrolyze specific proteins (keratinase, elastase, collagenase, etc.), or on the basis of their similarity to well-characterized proteinases such as pepsin, trypsin, chymotrypsin, or the mammalian cathepsins. The latter can often prove misleading if only a restricted number of properties are compared. The most satisfactory classification scheme is that proposed by Hartley (102) based on the catalytic mechanism: this forms the basis for the Enzyme Commission classification given in Table 1. There are four different types of proteinase, and these can be distinguished from one another on the basis of their sensitivity to various inhibitors (Table 1). Some commonly used inhibitors are not specific to one type of proteinase, however. For example, the chloromethyl ketone derivatives *N*-α-tosyl-L-lysine chloromethyl ketone (TLCK) and L-tosylamido-2-phenylethyl chloromethyl ketone (TPCK) and the microbial inhibitors leupeptin and antipain all inhibit some serine proteinases as well as many cysteine proteinases.

There is some overlap between the latter classification scheme and that based on pH dependence. The aspartic (carboxyl) proteinases are all active at acidic pHs, and in the absence of any supporting data, many acid proteinases have been assumed to be of the aspartic type. However, some have since been shown to be cysteine (thiol) proteinases, which are usually most active at slightly acidic pHs. Metalloproteinases are active around neutrality, and serine proteinases are usually most active at alkaline pHs. Indeed, the terms neutral proteinase and alkaline proteinase are often used synonymously for these two types. In this review, however, the terms acid, neutral, and alkaline proteinase are only used to refer to the pH optimum of an enzyme and do not imply anything about its catalytic mechanism.

The conclusions that can be made about the physiological role of a proteinase directly from its in vitro properties are, however, limited, since physiological substrates are not always used. The pH dependence may indicate something about the compartment in which the proteinase would operate (for example, intracellular acid proteinases might be expected to be vacuolar), but with non-physiological substrates considerable differences in pH optima are observed

for the hydrolysis of different proteins by the same enzyme. Casein hydrolysis is often optimal at higher pHs than hemoglobin hydrolysis; thus, a pH optimum may be as much a feature of the substrate as it is of the proteinase. Substrate specificity may indicate the range of proteolytic events in which a proteinase might be able to participate, but it cannot always be related directly to activity on the physiological substrates. Nevertheless, knowledge of the properties of a proteinase is important, especially since most organisms produce a number of different proteinases and it is essential that their activities can be distinguished from one another.

In the following sections, the properties of the proteinases of fungi (Eumycota), protozoa, and slime molds are discussed. For the fungal enzymes, only those properties which allow comparisons to be made between proteinases from different organisms or those which may be relevant to the physiological role of an enzyme are considered in detail. More comprehensive coverage is given for the protozoan and slime mold proteinases.

## FUNGI

A considerable amount of information is now available on the proteinases of fungi. Many surveys have been undertaken involving hundreds of species which have been screened for general proteinase activity on substrates such as casein or for more specific activities such as those of elastases, fibrinolytic enzymes, or milk-clotting enzymes. Only a proportion of the enzymes detected have been subjected to more detailed characterization. Most have properties consistent with aspartic, metallo-, serine, or cysteine proteinases. Many fungal exopeptidases have also been described, but discussion of their properties is outside the scope of this review.

Comparison of the proteolytic systems of the fungi is made difficult by the use in different laboratories of a variety of sometimes inadequately identified isolates of the same species, many unique to individual laboratories, by the variety of media and culture conditions employed, and by the use in some instances of crude commercial preparations as starting material for proteinase analysis (50). This variation is particularly apparent with one of the most studied species, *Aspergillus oryzae*. Nevertheless, fungal proteinases isolated from species representing different taxonomic groups do show a number of common features, which are discussed below.

For more detailed discussions of specific aspects, the reader should consult reviews on the yeast proteolytic system (361), proteinases of aspergilli (49), intracellular proteinases (116, 354), extracellular proteinases (50), and proteinase specificity (206) together with an earlier review in which detailed descriptions of the properties of some of the fungal proteinases are given (193).

### Aspartic Proteinases

Many fungi produce proteinases which are active at acidic pHs, and a large proportion of these have been shown to have properties consistent with aspartic proteinases. With some, labeled inhibitors have been used to isolate active-site peptides and to directly demonstrate the involvement of one or two aspartic acid residues (152, 185, 224). Recently, diazoketone reagents have been used for active-site spin labeling of the proteinase from *Rhizopus chinensis* (226). Not every acid proteinase is inhibited by the specific inhibitors pepstatin or *Streptomyces* pepsin inhibitor (S-PI), and a small but significant group of insensitive enzymes has now been reported: proteinase A of *Aspergillus niger* var. *macrosporus* (41, 124), proteinases A1, A2, and B of *Scytalidium lignicolum* (238), and the extracellular proteinase of the basidiomycete *Lentinus edodes* (332). Recently, extracellular proteinases from other basidiomycetes have also been reported to be S-PI insensitive (240). An extracellular proteinase from the basidiomycete *Sporotrichum dimorphosporum* has recently been described, but its sensitivity to inhibitors was not reported (328). An examination of the specificity of proteinases A1 and A2 of *S. lignicolum* suggests that the nature of the amino acid at the $P'_1$ position (as defined by Schechter and Berger [286]) is less important than in the other acid proteinases, in which a bulky amino acid is preferred (208).

In general, the pepsin inhibitor *p*-bromophenacyl bromide does not inhibit fungal acid proteinases. A number of them are sensitive to *N*-bromosuccinimide, $I_2$, and potassium permanganate, suggesting the possible involvement of tyrosine. The proteinases of *Penicillium caseicolum*, *Penicillium janthinellum* (penicillopepsin), *Penicillium roqueforti*, and *R. chinensis* are inactivated by butane-2,3-dione on exposure to light (92). This involves a reaction with tyrosine and tryptophan residues and in penicillopepsin with active-site residues. However, for the *P. roqueforti* enzyme the effect may be nonspecific.

Most of these proteinases have molecular weights in the range of 30,000 to 45,000, the exceptions being larger enzymes in *Podospora anserina* (161) and some carbohydrate-containing proteinases of *A. oryzae*, whose protein components have molecular weights between 29,000 and 34,000 (340); smaller proteinases

TABLE 1. Proteinase classification

| Type | Specific inhibitors; characteristic of enzyme type | Other inhibitors | Activators |
|---|---|---|---|
| Aspartic proteinases (EC 3.4.23) | Pepstatin<br>S-PI (acetyl pepstatin)<br>Diazoacetyl norleucine methyl ester<br>*N*-Diazoacetyl-*N*-'-2,4-dinitrophenylethylenediamine<br>Epoxy (*p*-nitrophenoxy) propane | | |
| Metalloproteinases (EC 3.4.24) | Chelating agents<br>EDTA, ethylene glycol-bis(β-aminoethyl ether)-N,N-tetraacetic acid<br>*o*-phenanthroline<br>8-hydroxyquinoline<br>α,α-dipyridyl<br>Phosphoramidon (not acid metalloproteinases) | | |
| Serine proteinases (EC 3.4.21) | PMSF<br>DIFP | TLCK<br>TPCK<br>Antipain<br>Leupeptin<br>Chymostatin | |
| Cysteine proteinases (EC 3.4.22) | Iodoacetamide, iodoacetate<br>Heavy metals<br>*N*-Ethyl maleimide | *p*-Chloromercuribenzoate (also inhibits some serine proteinases)<br>TLCK<br>TPCK<br>Antipain<br>Leupeptin<br>Chymostatin | Reducing agents<br>Cysteine<br>DTT<br>EDTA |

have been reported in *S. lignicolum* (238) and *A. niger* (41). All the reported isoelectric points are below pH 5.1, with the exception of that for the *S. dimorphosporum* proteinase (pH 7.4) (328), and the majority are between pH 3.4 and 4.6. Many of the purified proteinases contain carbohydrate, which is responsible for some proteinase heterogeneity. Detailed studies of the carbohydrate component have been made for *Mucor miehei* (270), *S. lignicolum* (217), and *A. oryzae* (339) proteinases.

Fungal acid proteinases are usually able to hydrolyze a range of native proteins, but the majority of them have little or no activity on small synthetic substrates. Earlier observations of such activity were often due to the presence of acid (serine) carboxypeptidases, which are now removed in modified purification schemes (see, e.g., references 122, 143, 218, 244 and 325). Many of the acid proteinases have milk-clotting activity, as they are able to cleave the Phe(105)—Met(106) bond of κ-casein, the bond cleaved by rennin. The pH optimum for milk clotting is usually closer to neutrality than that for proteinase activity. A synthetic rennin substrate based on the sequence around the Phe(105)—Met(106) bond is hydrolyzed by some milk-clotting fungal proteinases (119, 192).

The specificity of a number of the proteinases has been assessed by examining the hydrolysis of the oxidized B chain of insulin and of some other small, naturally occurring polypeptides (see references 121–123, 143, 252, and 325 for recent examples). Most of the fungal proteinases have broad specificity but preferentially hydrolyze peptide bonds between two bulky amino acids. With the exception of the pepstatin-insensitive proteinase A of *A. niger* (124) and the acid proteinase of *Candida albicans* (268), which is not inhibited by diazoacetyl norleucine methyl ester, all the fungal proteinases cleave the B chain Tyr(16)—Leu(17) and Phe(24)—Phe(25) bonds. The S-PI-insensitive *S. lignicolum* proteinases showed weaker activity on the former bond (239), which most other proteinases attack preferentially. Most of the proteinases also cleave the Leu(15)—Tyr(16) bond and to a lesser extent the Ala(14)—Leu(15) and Phe(25)—Tyr(26) bonds. Except for those proteinases unable to cleave the Tyr(16)—Leu(17) bond, only proteinase $I_a$ from *Pycnoporus coccineus* fails to cleave the Leu(15)—Tyr(16) bond, and it

has been suggested that this results from an inhibitory influence of the hydroxyl group of the tyrosine in the $P'_1$ site (122). This enzyme is also unable to cleave the Phe(25)—Tyr(26) bond.

An interesting property of many of the fungal acid proteinases is their ability to activate bovine trypsinogen (see references 20, 172, 251, 280, and 281 for recent examples). This was described first by Kunitz (159) in 1938 for an unidentified species of *Penicillium*. Morihara and Oka (207) reported a relationship between this trypsinogen kinase activity and the ability of proteinases to hydrolyze specific oligopeptides at bonds involving the carboxyl group of lysine, although lysine- and arginine-containing bonds in the insulin B chain are not cleaved. Activation involves the splitting of the Lys(6)—Ile(7) bond of trypsinogen, the bond cleaved during autocatalysis. Indeed, *A. oryzae* and *Aspergillus saitoi* enzymes have been used to study both trypsinogen (1, 276) and chymotrypsinogen activation (65, 295). Hog pepsinogen can be activated by *A. oryzae* proteinases (312). Not all of the fungal enzymes are able to activate trypsinogen, but it is interesting to note that proteinases from the protozoan *T. pyriformis* (65) and the cellular slime mold *D. discoideum* (235) have trypsinogen kinase activity.

Amino acid sequence analysis (93) and X-ray crystallographic analysis (120, 318, 327) of the proteinases of *R. chinensis*, *P. janthinellum*, *P. roqueforti*, and *Endothia parasitica* have revealed a considerable degree of homology between the fungal proteinases and mammalian aspartic proteinases including pepsin and rennin, suggesting that they all evolved from a common ancestral gene (326). The aspergillopepsin A from *Aspergillus awamori* is also being sequenced (151). Its active-site sequences show considerable homology with those of pepsin and penicillopepsin.

Fungal species which produce extracellular acid proteinases often acidify the medium in which they grow (50, 195). Since many of the enzymes are unstable above neutral pH, they are not found in cultures growing at neutral or alkaline pH.

### Metalloproteinases

Only a few examples of metalloproteinases have been reported in fungi, and most have been shown to be zinc-containing enzymes. Gripon et al. (90) have suggested that the enzymes of *P. caseicolum* and *P. roqueforti* and the neutral proteinase II of *A. oryzae* and *Aspergillus sojae* represent a distinct group of enzymes for which they suggest the name acid metalloproteinase. These have lower pH optima (5 to 6), lower molecular weights (19,000 to 20,000), and a different specificity with the oxidized insulin B chain from the thermolysin-like neutral metalloproteinases. The *Penicillium* proteinases are also insensitive to phosphoramidon, a specific neutral metalloproteinase inhibitor. The neutral proteinase I of *A. oryzae* and *A. sojae* have molecular weights of 41,000 and 42,000, respectively, and a pH optimum of 7 (221, 222, 291). The isoelectric points of *A. sojae* proteinases I and II are 4.7 and 4.2, respectively. The basidiomycete *Tricholoma columbetta* produces a low-molecular-weight neutral proteinase (165) which has some resemblance to the metalloproteinase of another basidiomycete, *Armillaria mellea* (180). However, this enzyme is not inhibited by ethylenediaminetetraacetate (EDTA), and at high concentrations the chelating agent enhances proteinase activity. This proteinase is not affected by diisopropylfluorophosphate (DIFP) or cysteine and is inhibited only by potassium cyanide. Further characterization is necessary to determine whether this enzyme represents a unique class of proteinase.

### Serine Proteinases

The production of alkaline proteinases has been described for fungi of all major taxonomic groups. Virtually all of those subjected to detailed characterization have been shown to be serine proteinases, since they are inhibited by either phenylmethylsulfonyl fluoride (PMSF) or DIFP. Many of them are also inhibited by some thiol reagents such as *p*-chloromercuribenzoate. This may reflect the close proximity of a cysteine residue to the active site. Binding of a bulky residue to this cysteine may indirectly interfere with substrate binding, as suggested for the serine carboxypeptidase of yeast, carboxypeptidase Y(12). It is unlikely that the cysteine participates in the catalytic mechanism, and in fact many of the fungal serine proteinases have no cysteine residues. Labeled DIFP has been used to locate active-site peptides from *Aspergillus* proteinases (204).

The proteinases are generally of low molecular weight, in the range of 18,500 to 35,000, and usually around 25,000. Larger enzymes have been reported in *A. niger* (30), *A. nidulans* (316), *Phycomyces blakesleeanus* (78), *Blastocladiella emersonii* (186), and *Blakeslea trispora* (88); the serine proteinase of the latter has a molecular weight of 126,000, the largest reported. The presence of carbohydrate in the purified enzyme has been reported in only a few instances. Most have low isoelectric points, between pH 4.4 and 6.2, but four, those of *Alternaria tenuissima* (133), *Fusarium* sp. (335), *N. crassa* extracellular proteinase (157), and *Tritirachium album* (proteinase K) (72), have isoelectric points of pH 8.9 or higher.

In general, the proteinases have a broad specificity. The cleavage patterns with oxidized insulin B chain are quite varied (see references 149 and 181 for recent examples). Most of the enzymes hydrolyze the Leu(15)—Tyr(16) and Phe(25)—Tyr(26) bonds. The Gln(4)—His(5), Glu(13)—Ala(14), Tyr(16)—Leu(17), and Tyr(26)—Thr(27) bonds are also cleaved by a high proportion of the fungal serine proteinases. There is only one bond in the insulin B chain, Pro(28)—Lys(29), which cannot be split by any of the enzymes reported to date.

The properties of the serine proteinases, including their cleavage patterns with the insulin B chain, have been used to examine the relationship between different species of *Aspergillus* (228, 342).

The ability to produce alkaline proteinases has been correlated with growth of fungi at neutral and alkaline pH (195).

### Cysteine Proteinases

Reports of the occurrence of cysteine proteinases in fungi are very limited. Since *p*-chloromercuribenzoate inhibits a number of the serine proteinases, proof that an enzyme is a cysteine proteinase must depend on the demonstration that it is sensitive to additional inhibitors such as iodoacetate and preferably that its activity can be enhanced by reducing agents such as cysteine and dithiothreitol (DTT) and possibly by EDTA.

Two isolates of *Trichosporon* sp. (94, 346) and the neuropathogenic fungus *Oidiodendron kalrai* (45) were reported to have intracellular enzymes optimally active at pH 6. An elastase activity of the dermatophytic fungus *Nannizzia fulva*, active at pH 8, was inhibited by *p*-chloromercuribenzoate and iodoacetate but was not enhanced by either cysteine or EDTA (274). Roberts and Doetsch (275) have described an enzyme from culture filtrates of a *Microsporum* species which was active at pH 6.8 and was enhanced by reducing agents. An extracellular enzyme that was inhibited by thiol reagents and enhanced by reducing agents has been reported in *A. oryzae* (135), and the same workers have described an extracellular collagenase from *Aspergillus sclerotionum* which may also be a cysteine proteinase, even though it was inhibited by EDTA (136).

### Summary

All four types of proteinase have been detected in fungi, although aspartic and serine proteinases predominate. Most of the reported proteinases are probably extracellular, the intracellular enzymes having received less attention. In some species, only one type of proteinase has been reported, but in most species at least two and sometimes three types of proteinase are produced, although not always under the same culture conditions. Multiple forms of proteinase of the same type may also be produced by the same organisms. Although the enzymes show some similarity to enzymes of the same type from other organisms, the fungal proteinases have, in general, a broader specificity than equivalent mammalian enzymes.

## PROTOZOA

Although reports of proteinase activity in protozoa date back to 1902 (see reference 215 for references to pre-1967 literature), only recently have more detailed characterizations of the proteolytic systems of many protozoa been undertaken. The majority of the species currently being studied are parasitic to humans or domestic animals or are closely related to such species, and research on protozoan proteinases has been stimulated by the idea that proteolysis may have essential roles in the host-parasite relationship. Since changes in proteolytic activity for different stages of the life cycle have been observed, it is important to note the stage of the parasite.

### Flagellates

Acid proteinase activity has been detected in both African (*Trypanosoma brucei*) and Latin American (*Trypanosoma cruzi*) species of trypanosomes. Although it was suggested initially that a *T. brucei rhodesiense* proteinase isolated from trypomastigotes was cathepsin D-like, that is, an aspartic proteinase (344), it has now been shown that the major proteinase from *T. brucei* bloodstream forms must be a cysteine proteinase, since activity is stimulated by both ethylene glycol-bis(β-aminoethyl ether)-*N,N*-tetraacetic acid and cysteine, inhibited by *p*-chloromercuribenzoate, and unaffected by pepstatin (311). The enzyme had an optimum pH around 4 for acid-denatured hemoglobin hydrolysis (310).

Similarly, the epimastigote proteinase from *T. cruzi* has been described as a cathepsin D (11), yet no evidence in support of this has been provided. Rather, with both of the purified proteinase preparations that have been described, activity is enhanced by reducing agents and inhibited by several thiol reagents. Rangel et al. (265) purified an epimastigote proteinase from strain Y which hydrolyzed hemoglobin and casein with optimum pH of 3 and 6, respectively. The purified preparation was found to contain one protein component on gel electrophoresis with a molecular weight of 60,000. Bongertz and Hungerer (29) have described a proteinase isolated from epimastigotes of strain D with a molecular weight of approximately 200,000. This enzyme had an isoelectric point of pH 5.5 and

contained 2% carbohydrate. It was active on a variety of substrates, including hemoglobin, α-*N*-benzoyl-DL-arginine-4 nitroanilide (Bz-Arg-pNA), α-*N*-benzoyl-L-argininamide, and α-*N*-benzoyl-L-arginine ethyl ester. The Bz-Arg-pNA hydrolysis was assayed at the relatively high pH of 8.5. Since Rangel et al. (265) reported that their enzyme had no arylamidase activity, it would appear that different enzymes have been purified. It is significant that Itow and Camargo (127), using the Y strain of *T. cruzi*, had previously shown that the esterase and amidase activities in epimastigote extracts differed from the proteinase activity (azocasein hydrolysis) on the basis of sensitivity to inhibition by various agents and temperature dependence.

Proteinase activity has also been reported in *Trypanosoma rangeli* (336), *Trypanosoma conorhini*, and *Trypanosoma mega* (38), although the latter had only a trace of activity with casein as substrate and low activity on Bz-Arg-pNA (38).

Four species of *Leishmania* have been shown to have proteinase activity, although that of *Leishmania donovani* (38, 311) and *Leishmania braziliensis* (38) has not been characterized. For *Leishmania mexicana mexicana*, North and Coombs (233) have used electrophoresis on polyacrylamide gels containing denatured hemoglobin to demonstrate multiple forms of proteinase active at pH 4.0 in both the promastigotes and amastigotes. Amastigote extracts have four major proteinases with six minor forms. In promastigotes, the pattern is both qualitatively and quantitatively different. That promastigotes have less proteinase activity than amastigotes has also been shown by azocasein hydrolysis at pHs in the range of 2.5 to 8.5 (58). Both types of analysis have shown that the proteinases are stimulated by DTT and are sensitive to inhibitors of cysteine proteinases, including iodoacetate, TLCK, antipain, and leupeptin. Phenanthroline, normally considered to be an inhibitor of metalloproteinases, also reduced the proteinase activity. Interestingly, the proteinase pattern observed for *L. mexicana mexicana* was not found with the reptilian parasite, *Leishmania tarentolae* (M. J. North and G. H. Coombs, unpublished observations). In this species, only two proteinase bands were detected on hemoglobin gels, and the enzymes had a considerably lower electrophoretic mobility than the major proteinases of *L. mexicana mexicana*. Although their enhancement by DTT and inhibitor sensitivity suggested that they were also cysteine proteinases, the difference in proteinase pattern lends some support to the idea that the reptilian leishmanias may not be very closely related to the mammalian species.

*Crithidia* are insect flagellates that have been used as models for the salivarian trypanosomes. Their proteinase activity is lower than that of other flagellates. Camargo et al. (38) reported negative caseinase activity in five species of *Crithidia* which could hydrolyze Bz-Arg-pNA. However, Eeckhout (73) described an enzyme from *Crithidia lucilae* which hydrolyzed hemoglobin at acid pH, and other reports of proteinases active on hemoglobin (311) and azocasein (58) have since appeared. A preliminary analysis on hemoglobin gels of the activity from *Crithidia fasciculata* has indicated two forms of cysteine proteinase active at pH 4 (M. J. North and G. H. Coombs, unpublished observations). Three species of *Leptomonas* and four of *Herpetomonas* were reported to have no caseinase activity (although Bz-Arg-pNA hydrolysis was noted) (38), but Coombs (58) has demonstrated low activity on azocasein at neutral pH in extracts from *Leptomonas ctenocephali*, *Herpetomonas muscarum*, and *Herpetomonas ingenoplastis*.

Peptidase activity has been detected in a number of species of kinetoplastida. Six peptidases which correspond closely to those in human erythrocytes have been identified in bloodstream forms of *T. brucei* by starch gel electrophoresis (174). In *T. brucei gambiense*, peptidase polymorphism has been demonstrated (86). The peptidase pattern of *T. brucei* does differ for different stages of the life cycle (A. Tait, personal communication). Peptidase activity has also been detected in *L. mexicana mexicana*, and as with the proteinases there are qualitative and quantitative differences between the peptidases of amastigotes and promastigotes (A. Tait and G. H. Coombs, personal communication). *C. fasciculata* possesses arylamidases which hydrolyze the β-naphthylamides of leucine, arginine, lysine, and glutamic acid (250).

McLaughlin and Müller (198) have purified a proteinase from *Tritrichomonas foetus*, a flagellate parasite of the genitourinary tract of cattle. They suggest that the enzyme is responsible for the hydrolysis of denatured hemoglobin, azocasein, and α-*N*-benzoyl-L-argininamide by cell homogenates. It has a molecular weight of between 17,500 and 20,000. With α-benzoyl-DL-arginine-2 naphthylamide (Bz-Arg-2Nap) as substrate, the optimum pH was 5.5; with other substrates, the optimum pH was 6.5 to 7.0. Its activity was blocked by a number of cysteine proteinase inhibitors but not by pepstatin. An electrophoretic analysis of the proteinases of the human parasite *Trichomonas vaginalis* has revealed a more complex proteolytic system in this species (60). At least seven forms of proteinase were active on hemoglobin at pH 4.0, three of which were as active at pH 6.0. Only one of the other four could be detected at pH 6.0. All were activated by DTT and inhibited by cysteine

proteinase inhibitors. However, the three most active at pH 6 were more dependent on DTT and were also more sensitive to inhibition than those active only at pH 4. At least two aminopeptidases active on glycyl-L-phenylalanine-β-naphthylamide are present in *T. foetus* (198).

*Monocercomonas* sp., a primitive trichomonad, has a proteinase active on hemoglobin at pH 7 (184).

Proteinases have also been reported in phytoflagellates. Those of *Ochramonas danica* (58) and *Ochramonas malhamensis* (141) have not been characterized, but the proteolytic system of *Euglena gracilis* has been studied in some detail. Bertini et al. (21) described an uncharacterized acid proteinase (cathepsin). An analysis by Zeldin and co-workers (373, 374) revealed two classes of proteinase, one active at neutral pH and the other active at pH 3.5. Unusually for a proteinase active at low pH, the latter class was inhibited by PMSF. A serine proteinase associated with *Euglena* ribosomes has been described (166). Intracellular proteinase was also detected by Nakano et al. (225) and was optimal at pH 7.3: esterase and amidase activities in the same extracts had lower pH optima. The proteinase activity was inhibited by the serine proteinase inhibitor DIFP. However, an enzyme with proteinase, amidase, and esterase activity has been isolated from culture medium and purified (225), and this enzyme is severely inhibited by DIFP and to some degree by phenanthroline, EDTA, and *p*-chloromercuribenzoate. Optimal pHs for the three types of activity were 7.3, 7.0, and 6.3, respectively. The enzyme had a molecular weight of 41,000 and an isoelectric point of pH 8.3.

More is known about the intracellular peptidases of *E. gracilis*. Six major types of aminopeptidase and an acid carboxypeptidase have been described, many of which have been purified and characterized (269, 292, 293). Further multiplicity has been revealed on purification (294). A detailed discussion of these enzymes is outside the scope of this review.

### Amoebae

Although some early reports exist on the proteolytic enzymes of free-living amoebae (see reference 215), the organism most studied is the parasite *Entamoeba histolytica*. Preliminary attempts to characterize its proteinases revealed caseinase and gelatinase activities at pH 5 to 8 (101, 223, 229). Jarumilinta and Maegraith (129, 130) showed that both the trophozoites and extracts prepared from them were able to hydrolyze a number of proteins and synthetic substrates. The enzyme responsible was suggested to be trypsin-like on the basis of its specificity. The authors also reported peptidase activity. McLaughlin and Faubert (197) have recently undertaken the partial purification of two proteinases which can be separated by chromatography on diethylaminoethyl Bio-Gel A. One, active on azocasein at pH 6.0, was inhibited by cysteine proteinase inhibitors such as iodoacetamide and *p*-chloromercuribenzoate and was activated by DTT and cysteine. The second enzyme was active at pH 3 with hemoglobin or serum albumin as substrates and was insensitive to the thiol agents and all other inhibitors that were used. However, with the exception of bromophenacyl bromide, an inhibitor of rather limited specificity, no aspartic proteinase inhibitors were tested; thus, it is not known to which class this acid proteinase belongs.

Proteinase activity has been reported in various species of *Acanthamoeba*, Jarumilinta and Maegraith (129) described caseinase and gelatinase activities at pH 7.6; in the most recent report, Auriault and Desmazeaud (10) described acid proteinase in extracts of *Acanthamoeba culbertsoni* and *Acanthamoeba rhysodes*, which hydrolyze hemoglobin at pH 3.8. No activity was detected at pH 6.5 or 8.5 with azocasein. Both extracellular and intracellular aminopeptidases were present (10).

Two proteinases with pH optima of 5.0 and 9.5 are released by *Hartmanella culbertsoni* during excystment (137). One of these has been partially purified and characterized (139). The purified enzyme had a molecular weight of 21,400 and a pH optimum between 8 and 9 with casein as substrate. It was not affected by thiol reagents but was inhibited by PMSF and is believed to be a serine proteinase.

### Sporozoa

The species most studied are those of the genus *Plasmodium*, the malarial parasites. However, successful analysis of their proteinases has been hindered by the difficulty of obtaining samples of parasites which are free of host cell material, particularly erythrocyte membranes. Since the membranes themselves have acid (216, 260, 267) and neutral (334) proteinases, it is essential that parasite and host activities be distinguished. Many workers have compared the properties of proteinases in parasite preparations with those of enzymes in similar preparations from uninfected blood and have reported higher levels of activity in the former. However, little work has been carried out to determine the effect that the presence of parasites might have on the recovery of erythrocyte proteinases. In many cases, therefore, the origin of the proteinases described is in doubt.

Proteolytic activity in malarial parasites was first reported by Moulder and Evans (212) in 1946 for *Plasmodium gallinaceum*, a chicken

parasite. The first attempts to characterize *Plasmodium* proteinases were made by Cook et al. (56), who examined both rodent (*Plasmodium berghei*) and monkey (*Plasmodium knowlesi*) species. They reported soluble proteolytic activity with pH optima (4 and 8) for hemoglobin hydrolysis. The alkaline activity was inhibited by DIFP, EDTA, and 8-hydroxyquinoline, and it was concluded that the enzyme was not derived from host material since erythrocyte activity was DIFP insensitive. More recently, the occurrence of DIFP- and EDTA-sensitive proteinases has been reported in human and ovine erythrocyte membranes (290, 334). Cook et al. (57) confirmed the presence of a soluble alkaline proteinase in preparations of *P. knowlesi*. Chan and Lee (40) found three peaks of alkaline proteinase activity in samples of *P. berghei* subjected to ion-exchange chromatography, but further characterization has not been undertaken. Sherman (297) has pointed out that, in view of the sensitivity of the activity to EDTA (56), the use of a chelating agent would prevent detection of alkaline proteinases in *Plasmodium* preparations.

Proteinases active at lower pHs are also present in parasite preparations. Levy and co-workers have described them for *Plasmodium falciparum* and *P. knowlesi* (178) and *P. berghei*. (175, 176). The proteinase activity of infected cells was solubilized less readily than that of the control uninfected cells. For each species, it was found that the optimum pH for hemoglobin digestion was marginally higher for the samples containing parasites (pH 3 to 3.6) than it was for the corresponding host cells (pH 2 to 2.5 for the monkey cells; pH 2.5 to 3.5 for the mouse cells). Activity in samples of parasites was inhibited by pepstatin and chymostatin and to a lesser extent by leupeptin and antipain. It was unaffected by thiol agents. Activity from infected (with *P. berghei*) and uninfected mouse erythrocytes was also inhibited by PMSF, although this was not so for the monkey activities. Mahoney and Eaton (190) have described a proteinase in samples of *P. berghei* that is active on hemoglobin at pH 6. It was inhibited by pepstatin but not by PMSF, ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*-tetraacetic acid, or *N*-ethylmaleimide. The enzyme was apparently unstable, and this precluded its purification.

Attempts to separate acid proteinases in *Plasmodium* samples have been made. By using polyacrylamide gel electrophoresis followed by an imprint-digest method, Hempelmann and Wilson (108) compared the patterns of hemoglobin-digesting proteinases in preparations of *P. knowlesi* schizonts and of uninfected monkey erythrocytes. Whereas the latter had only one major activity at pH 3.2, with two minor activities, one of which was probably of platelet or lymphocyte origin, the parasite preparation was characterized by three additional proteinases. Two of these were also observed when merozoite extracts were used. The properties of the proteinases have not been reported, however. North and Coombs (unpublished observations) have used electrophoresis on hemoglobin gels (233) to analyze the proteinases in samples containing the rodent parasite *Plasmodium chabaudi*. Three bands of activity were detected at pH 4.0, two of which correspond to bands obtained with samples from uninfected blood and have highest activity in those parasite preparations most contaminated with host cell material. An analysis of their sensitivity to inhibitors showed a striking similarity to those described for the acid proteinases of human and rabbit erythrocyte membranes (260, 261, 267). This includes the somewhat unexpected inhibition of one of them by DTT and their sensitivity to TPCK and mercuric ions. It is significant that, whereas these two enzymes were also inhibited by pepstatin, the third enzyme, detected only in parasite preparations, was apparently pepstatin insensitive. The possibility that a major proteinase activity is lost during electrophoresis cannot be ruled out, particularly in view of the reported instability of the proteinase in preparations of *P. knowlesi* (190). Nevertheless, the results indicate that active proteinases of erythrocyte origin were certainly present in these *Plasmodium* preparations.

The problem of differentiating parasite proteinases from erythrocyte proteinases is also encountered with another intraerythrocytic parasite, *Babesia*. Aissi and Charet (4) have analyzed samples of *Babesia hylomysci* by electrophoretic methods similar to those used by Hempelmann and Wilson for *P. knowlesi* (108) and by North and Coombs for *P. chabaudi* (see above). Two proteinase bands were observed for both uninfected blood cells and parasite samples. The results suggest that these bands, which appear to be identical to the two observed by North and Coombs (see above), were due to erythrocyte enzymes. This is also suggested by similarities in proteinase properties, including a molecular weight of 100,000 (4). Proteolytic activity has also been reported by Wright and Goodger (366), who described esterases (hydrolysis of tosyl-L-arginine methyl ester at pH 4 to 5 and 8 to 9) and a proteinase (hydrolysis of hemoglobin at pH 5) in sonicated extracts of *Babesia argentina* parasite-stroma suspensions. *Babesia bigemina* preparations had strong proteinase activity but only weak esterase. Activity on tosyl-L-arginine methyl ester has also been detected in samples prepared from *Babesia bovis* (367).

Charet and co-workers (4, 42) have also examined the aminopeptidases of *Plasmodium yoelii nigeriensis*, *P. chaboudi*, and *B. hylomysci* and found similarities between the *Plasmodium* and *Babesia* enzymes. Interestingly, these enzymes are inhibited by antimalarial drugs such as chloroquine, quinacrine, primaquine, and quinine.

In *Eimeria tenella*, a chicken parasite, only low levels of proteinase activity have been detected at pH 4 (347). No activity was detectable at pH 7. Despite its apparently low pH optimum, this activity was inhibited by PMSF. This organism has much higher levels of aminopeptidase activity (347), as has *Eimeria nieshulzi* (43). The aminopeptidase activity of the latter species is also sensitive to antimalarial drugs (43).

### Ciliates

Proteinase activity was first studied in *T. pyriformis* by Lawrie (168, 169). Cell extracts digested casein, gelatin, and α-glutalin with optimal activity on gelatin at pH 6.0. Viswanatha and Liener (345) obtained a crystalline mercury derivative of a proteinase which digested hemoglobin at pH 5 to 6. The enzyme, which also hydrolyzed *N*-carbobenzoxy-glutamyl-tyrosine at pH 4.0, was activated by cysteine. Dickie and Liener (67, 68) purified and partially characterized three proteinases from *T. pyriformis* W, one intracellular enzyme and two extracellular enzymes, one of which was obtained from cultures grown without glucose and one from cultures grown with glucose. The proteinases had molecular weights of 29,300, 10,400, and 17,500, respectively, and optimum pHs for activity on urea-denatured hemoglobin of 5.5, 6.5 to 7.0, and 7.0 to 8.0, respectively. Of the synthetic substrates tested, only α-*N*-benzoyl-L-arginine ethyl ester was hydrolyzed by the intracellular enzyme and to a lesser extent by the extracellular enzyme from glucose cultures. The other extracellular enzyme had no esterase activity. The proteinases exhibited broad specificity on bovine insulin cleaving the B chain at the following bonds: Tyr(16)—Leu(17), Tyr(26)—Thr(27), and Thr(27)—Pro(28). This pattern is different from that reported for any of the fungal proteinases. More recently, Levy et al. (179) have described intracellular activities with azocasein, hemoglobin, and Bz-Arg-2Nap as substrates at pH 8.0, 3.6, and 6.0, respectively. Five active species can be separated by ion-exchange chromatography, two of which are active on all three substrates and three only on Bz-Arg-2Nap. Further purification and characterization indicated that the same enzyme was responsible for hydrolyzing both azocasein and hemoglobin. From gel filtration on Sephadex G-100, it was shown to have a molecular weight of 25,000, similar to that reported by Dickie and Liener (67). In cell extracts, the activity was enhanced by reducing agents and EDTA, and in purified preparations there was an absolute requirement for a reducing agent for azocasein hydrolysis. The activity was inhibited by cysteine proteinase inhibitors, including chymostatin, leupeptin, and antipain, but was unaffected by pepstatin and PMSF. Hydrolysis of Bz-Arg-2Nap in purified fractions was more sensitive to the cysteine proteinase inhibitors than the proteinase activity. An acid proteinase from *Tetrahymena* has been reported to activate trypsinogen (65), but it is not known whether the enzyme responsible was the same as that described by Levy et al. (179).

Further evidence for multiple forms of proteinase has been obtained by Blum (26), who resolved at least three and probably five secreted proteinases by using ion-exchange chromatography. The enzymes were active on hemoglobin at pH 3.4. No detailed characterization was undertaken, but Blum (25) had earlier reported that the secreted activity was inhibited by chymostatin, leupeptin, and antipain. Approximately 25% of the activity remained, even with high concentrations of inhibitor, suggesting the occurrence of more than one type of proteinase. Pepstatin had no effect, however.

Peptidase activity has been detected in *Tetrahymena*, the most recent being that of Zdanowski and Rasmussen (372), who described peptidases in the cytoplasm and on the outer cell surface of *Tetrahymena thermophila*.

Although *Paramecium* proteinases were first reported in 1903 (202), further study has been limited. The only recent reports concern a proteinase which affects the structure of the cell surface immobilization antigen. In *Paramecium aurelia*, the proteinase can be separated from the immobilization antigen by ion-exchange chromatography and is activated by mercaptoethanol and DTT, suggesting that it is a cysteine proteinase (308). A similar report has been made for *Paramecium primaurelia* and *Pseudomicrothorax dubius*, in which a proteinase of 25,000 molecular weight is believed to be involved (28). The finding that this proteinase can be inactivated by a PMSF-sensitive mechanism implies the existence of a further proteolytic enzyme.

*Entodinium ecaudatum* has an enzyme which hydrolyzes α-*N*-benzoyl-L-argininamide with optimum activity at pH 6.5 to 7.0 (2). It is enhanced by cysteine. Peptidase activity has also been detected at neutral pH with glycyl-L-leucine as substrate (2).

### Summary

Although there has been an increase in our knowledge of protozoan proteinases in the last few years, only a very small number have been purified and subjected to detailed characteriza-

tion. Nevertheless, some general features are apparent. The most notable is the frequency with which cysteine proteinases have been detected, in contrast to the few examples of this type reported in fungi. This probably reflects the predominantly intracellular location of the protozoan proteinases. Bongertz and Hungerer (29) have suggested that the susceptibility of the proteinase from epimastigotes of *T. cruzi* strain D to oxidation may allow the enzyme to be active for just that period required for penetration of the host cell by the parasite. Second, with the exception of the *Plasmodium* acid proteinases, the origin of which must still be in doubt, there is no evidence for pepstatin-sensitive aspartic proteinases, despite the low optimal pHs of many of the enzymes. Tang's (326) proposal that the *Tetrahymena* acid proteinase is part of an evolutionary family including fungal aspartic proteinases and mammalian enzymes such as pepsin, rennin, and cathepsin D seems unlikely in view of the similarity of the *Tetrahymena* enzyme to cysteine proteinases. One proteinase whose further characterization would be of great interest is the acid proteinase of *E. histolytica* (198), which is not a cysteine enzyme. The effect of pepstatin on the activity of this enzyme has not been tested. Metalloproteinases have not been demonstrated in protozoa, although phenanthroline, a metalloproteinase inhibitor, has an effect on many flagellate proteinases (58, 233; M. J. North and G. H. Coombs, unpublished observations) which by all other criteria would be considered to be cysteine enzymes. Serine proteinases have been reported in *E. gracilis* (166, 225) and *H. culbertsoni* (139), and the proteinase of *E. tenella* is also PMSF sensitive (347), although it is active at an acid pH. To date, only proteins and simple synthetic substrates (amino acid derivatives) have been used to detect and analyze proteinase activity, and it may be necessary to employ more specific synthetic substrates to reveal additional proteolytic enzymes.

## SLIME MOLDS

The Mycetozoa or slime molds represent a unique group of organisms with some fungal characteristics and some protozoan characteristics but with an overall life cycle that has no equivalent among the fungi or protozoa. Consequently, it is better to consider them separately, although it is of interest to compare their proteinases with those of the fungi and protozoa to see whether this provides any information about their relationship to other lower eucaryotes.

### Acellular Slime Molds

The proteinases of two species of acellular slime mold have been examined. In *P. polycephalum*, extracellular and intracellular forms have been detected. Farr et al. (75) found three proteinases in the culture fluid of microplasmodia and purified the major one, proteinase II. It had a molecular weight of 30,000 to 35,000 and an isoelectric point of pH 4.6. With azocoll as substrate, it had a pH optimum of 4.5 to 5.0 and was inhibited not only by thiol reagents such as *p*-hydroxymercuribenzoate and iodoacetamide (albeit at a very high concentration of 50 mM) but also by chelating agents and reducing agents. Aspartic proteinase inhibitors were not tested, and it is not clear to which class this proteinase belongs. An interesting property of this enzyme is its milk-clotting activity. Like rennin, proteinase II cleaved the Phe(105)—Met(106) bond of κ-casein. By using isoelectric focusing, Haars et al. (98) separated four proteinases, active on azohemoglobin at pH 4.1, from extracts of growing amoebae. The isoelectric points were in the range of pH 3.5 to 5.0. With growing plasmodia, there were nine enzyme forms. Only one extracellular proteinase was detected, but since it has not been characterized, its relationship to the rennin-like proteinase II is not known.

Multiple proteinases active on azocoll at pH 7 have been found in extracts prepared from growing haploid cells and dormant microcysts of *Physarum flavicomum* (110). Enzymes with isoelectric points ranging from pH 3.3 to 9.8 were detected after isoelectric focusing. Asgari and Henney (8) have also reported a proteinase activity which may be an inherent part of the extracellular slime of this species.

*Physarum* aminopeptidases have also been analyzed. Polanshek et al. (258) have described 12 electrophoretically distinct enzymes from *P. polycephalum* capable of hydrolyzing L-leucine-β-naphthylamide. Hoffman and Hüttermann (113) also detected multiple aminopeptidases by using 10 4-nitroanilide derivatives of amino acids. Multiple leucine aminopeptidases have also been described in *P. flavicomum* (110). Franke and Berry (82) chose leucine aminopeptidase as one of the enzymes used for a taxonomic study of the order Physarales and showed that all but one species, *Fuligo cinerea*, had some activity.

### Cellular Slime Molds

A more detailed characterization of proteinases has been achieved with the cellular slime molds. Most of the work has been undertaken with one species, *D. discoideum*, and its intracellular proteolytic enzymes. Proteinase activity was first reported in 1969 (322), and in 1970 Weiner and Ashworth (353) showed that myxamoebae have a lysosomal activity which hydrolyzes hemoglobin at acid pHs. It was optimal at pH 2 or below. Subsequently, it has been demonstrated that multiple forms of proteinase are

active in the acid pH range. Fong and co-workers (79, 80) have provided evidence for two types of enzyme, one active on protein substrates at pH 2.75 and the other active on Bz-Arg-pNA and Bz-Arg-2Nap, as well as proteins at pH 5.5. On the basis of their sensitivity to inhibitors, it was suggested that these proteinases correspond to cathepsin D and cathepsin B, respectively. By using electrophoresis on polyacrylamide gels containing denatured hemoglobin, North and Harwood (234) identified eight proteinases active below pH 5. These could be divided into two groups according to inhibitor sensitivity. The four most mobile enzymes were sensitive to a number of cysteine proteinase inhibitors and are probably responsible for the cathepsin B activity (80). The slower enzymes were sensitive only to mercuric chloride. One of these, proteinase E, was the most active proteinase at all pHs below 5 (234). This enzyme has been purified up to 200-fold (235). It had a molecular weight of approximately 53,000 and hydrolyzed a range of proteins with an optimum pH of 2.0 to 3.5. Like many fungal acid proteinases, it can activate bovine trypsinogen at acid pH. It was inactivated by diazoacetyl norleucine methyl ester at pH 4.8, although this can only be demonstrated in purified and salt-free preparations, and by epoxy(*p*-nitrophenoxy)propane at pH 3. It was insensitive to pepstatin, however. It was also inhibited by mercuric chloride and organomercurials but was insensitive to other cysteine proteinase inhibitors such as iodoacetate, TLCK, leupeptin, and antipain. It is therefore considered to be a pepstatin-insensitive aspartic proteinase which may possess a reactive, nonessential cysteine residue to which mercurials bind, inactivating the enzyme indirectly. Proteinase E probably contributes most of the cathepsin D activity in cell extracts. However, Fong and co-workers (79, 80) reported that the cathepsin D activity was pepstatin sensitive, in contrast to the findings of North's group (230, 234, 235). The reason for this difference is not known.

A second proteinase, proteinase I, has been purified from *D. discoideum* myxamoebae. Gustafson and Thon (97) have described an enzyme of unspecified molecular weight but consisting of three polypeptides, A (approximate molecular weight 34,500), C (approximate molecular weight 10,400), and B (unknown but intermediate molecular weight). The enzyme had proteinase activity on azocasein (pH optimum 4) and gelatin and esterase activity with α-*N*-carbobenzoxy-L-lysine *p*-nitrophenol ester (pH optimum 5.5). The latter substrate was used for routine assays of the enzyme, although it has not been reported whether other enzymes in cell extracts can hydrolyze the substrate. The purified enzyme also inactivated endogenous enzymes, including uridine diphosphate(UDP)-glucose pyrophosphorylase, at neutral pH in vitro. It was inhibited by iodoacetate, cystamine, and TLCK and required DTT for full activity, indicating that it was a cysteine proteinase. Although detailed comparisons with the cathepsin B activity (80) have not been made, its properties (pH dependence and inhibitor sensitivity) suggest that proteinase I makes a major contribution to the cathepsin B activity of cell extracts.

Further analysis of proteinase I in Gustafson's laboratory (95) revealed the presence of *N*-acetylglucosamine-1-phosphate in the enzyme, and since *O*-phosphorylserine was released after acid hydrolysis, it was concluded that the majority of the *N*-acetylglucosamine-l-phosphate residues were esterified to peptidyl serines. This moiety is believed to be responsible for a common antigenic determinant in proteinase I and another hydrolytic enzyme from *D. discoideum*, β-*N*-acetylglucosaminidase (96), although Knecht and Dimond (147) suggest that the situation may be more complex than suggested by Gustafson and Milner (96).

Further proteolytic activities have recently been detected in *D. discoideum* with $NH_2$-terminal-blocked tri- and tetrapeptide 4-nitroanilides (232). Some were due to particulate enzymes active below pH 7, and others were due to soluble enzymes active at pH 7.5.

Multiple proteinases are also present in four other species of cellular slime mold, *Dictyostelium mucoroides*, *Dictyostelium purpureum*, *Polyphondylium violaceum*, and *Polysphondylium pallidum* (150). As in *D. discoideum*, two distinct groups of proteinase may be distinguished on the basis of inhibitor sensitivity. Only one proteinase, proteinase D of *P. pallidum*, was inhibited by pepstatin.

Proteinase activity has also been reported in germinating microcysts of *P. pallidum* (242). Two activities were distinguished on the basis of pH dependence and changes in their relative levels during germination. The enzymes responsible hydrolyzed casein, proteinase A at pH 3.5, and proteinase B at pH 6. It is not yet known whether these correspond to any of the proteinases detected by gel electrophoresis in microcyst extracts (150).

There has been little study of the peptidases of the cellular slime molds. Firtel and Brackenbury (77) partially purified a leucine aminopeptidase active on L-leucine-*p*-nitroanilide from *D. discoideum*. Other aminopeptidases have been detected (80; G. Hughes and M. J. North, unpublished observations) but await characterization. No carboxypeptidase activity has yet been described, but carboxypeptidases could be responsible for some of the activities observed with

chromogenic peptide substrates (232). O'Day (241) has reported leucine aminopeptidase activity in germinating microcysts of *P. pallidium*.

**Summary**

Few general conclusions can be made at present about the proteolytic systems of the slime molds. In all species examined, there is evidence for multiple proteinases, but few have been subjected to detailed characterization. In the cellular slime molds, cysteine proteinases are active in the acid pH range, a common feature in protozoan species. However, other acid proteinases which are not cysteine enzymes are also present. It would be of interest to know whether these have any similarity to the acid proteinase of *E. histolytica*, an organism which has, in addition, a cysteine proteinase (197). Serine and metalloproteinases have not been reported in the slime molds, but a wider range of substrates must be tested before their presence can be ruled out. It may be significant that PMSF has been used successfully to protect a number of proteins from inactivation during their preparation from extracts of *D. discoideum*.

## PROTEINASE INHIBITORS

Any assessment of the role of proteinases within living cells must take into account the possibility that inhibitors of endogenous proteolytic enzymes may also be present. Table 2 lists those organisms from which proteinase inhibitors have been isolated or in which their presence is suspected. Some of the inhibitors have not been shown to have activity on endogenous proteinases, but others, particularly those from yeast, are very specific and inhibit one type of endogenous proteinase or peptidase only.

The best characterized inhibitors are those of *S. cerevisiae* and *Saccharomyces carlsbergensis*, and detailed discussions have appeared in recent reviews by Wolf and Holzer (361) and Wolf (354). The inhibitors are specific for the aspartic proteinase A, the serine proteinase B, or carboxypeptidase Y and bind to the enzymes to form 1:1 complexes. Beck et al. (18) have shown that proteinase A and its inhibitor are two independently synthesized polypeptide chains. Two forms (isoinhibitors) of both the proteinase A inhibitor ($I^A$) and proteinase B inhibitor ($I^B$) have been found, and the exact isoinhibitor pattern depends on whether they are isolated from *S. cerevisiae*, *S. carlsbergensis*, or baker's yeast. $I^A$ and $I^B$ are small proteins and are acid and heat stable. Examples of each type have been sequenced (24, 191), but, unlike heat-stable proteinase inhibitors from other sources, they do not possess disulfide bridges. The carboxypeptidase Y inhibitor ($I^C$) is a larger protein and is neither heat nor acid stable.

The yeast inhibitors are inactivated by proteinases A and B, although neither proteinase inactivates its own inhibitors (170, 282). The degradation of the inhibitors in cell extracts leads to the activation of proteinases A and B and carboxypeptidase Y during storage at pH 5 (282). Activation of fungal proteinase activity has been observed in other species, including *A. nidulans* (47) and *A. niger* (315). Although for both species it was originally suggested that this might involve the activation of a zymogen form of the proteinase, recent observations on the *A. nidulans* proteinases indicate that activation can be accounted for in terms of the loss of proteinase inhibitor (7). Zymogen forms of proteinase have been reported in *N. crassa* (209), but it is not known whether the data might also be explained in terms of a proteinase-inhibitor complex.

Within yeast cells, the inhibitors are cytosolic and thus separated from the vacuolar proteinases (see below). Since they are normally present in excess over proteinase activity, it is possible that the inhibitors function as a safety device against unwanted proteinase action due, for example, to vacuolar leakage. Beck et al. (18) have recently studied a yeast mutant with altered regulation of proteinase A inhibitor activity. The growth of the mutant, which has a 70% reduction in proteinase A inhibitor activity, is sensitive to temperature and pH. Unlike wild-type cells, mutant cells have an excess of proteinase over inhibitor, but this does not lead to any detectable changes in overall protein degradation. However, two proteins which are susceptible to proteinase A in vitro, proteinase B inhibitor and tryptophan synthase, show enhanced loss of activity in vivo under restrictive growth conditions. Although these experiments do suggest a vital cellular function, most questions about the in vivo role of the inhibitors remain unanswered. If they represent part of a general safety mechanism against leakage of vacuolar proteinases, their widespread distribution within the protozoa might be anticipated. To date, inhibitors have only been reported in *Paramecium* and *Tetrahymena* (171).

## PROTEINASE LOCALIZATION

It is evident that the role of a proteinase must be directly related to its location. Many of the fungal proteinases have been recovered from culture filtrates, although this need not indicate that they are truly extracellular since some were isolated from cultures in which the cells had undergone autolysis (see reference 49). However, for at least some fungi, it has been shown that enzymes are released during nonautolytic growth (50). The mechanism of release remains somewhat speculative. An early suggestion that

TABLE 2. Proteinase and peptidase inhibitors of eucaryotic microorganisms

| Species | Enzyme inhibited | Comment | Reference |
|---|---|---|---|
| *Aspergillus japonica* | Papain,[a] cysteine proteinases[a] | E64, epoxysuccinate derivative | 99 |
| *Aspergillus nidulans* | Proteinase A | Heat stable, nondialyzable | 7 |
| *Aspergillus oryzae* | Fungal alkaline proteinases, papain[a] | | 194 |
| *Candida albicans* | Chitin synthetase activator | Heat stable, trypsin sensitive | 32 |
| *Cephalosporium* sp. | Trypsin[a] | | 338 |
| *Euglena gracilis* | Aminopeptidase | Protein | 19 |
| *Histoplasma capsulatum* | | Protein P6, appears coincident with decrease in proteolytic activity | 314 |
| *Mucor rouxii* | Chitin synthetase activator | Heat stable, nondialyzable | 199 |
| *Neurospora crassa* | Proteinase I (alkaline proteinase) | Heat-stable proteins $I_2$ and $I_B$ | 157 |
| | Carboxypeptidase | Heat-stable protein $I_2$ | 157 |
| | Aminopeptidases $A_1$,$A_2$ | Heat-stable proteins $I_1$,$I_2$,$I_3$,$I_B$ | 157 |
| *Neurospora* sp. | Papain,[a] pronase,[a] trypsin[a] | Heat and alkali sensitive, high molecular weight | 247 |
| *Paramecium caudatum* | Cathepsins B[a] and H[a] | Heat stable | 171 |
| *Phycomyces blakesleeanus* | Proteinases B-CM, B-DI, and B-DII | Heat-stable, acid-resistant proteins I and II | 78 |
| *Penicillium cyclopium* | Penicillia acid proteinases | Poly(L)-malic acid | 299 |
| *Rhodotorula glutinis* | Carboxypeptidase R | Heat-stable protein | 111 |
| *Saccharomyces carlsbergensis* and *Saccharomyces cerevisiae* | Proteinase A | Heat-stable protein I^2,I^3 | 36 |
| | Proteinase B | Heat-stable proteins $I^B1$,$I^B2$ | 36 |
| | Carboxypeptidase Y | Heat- and acid-labile protein $I^C$ | 36 |
| *Tetrahymena pyriformis* | Cathepsins B[a] and H[a], *Tetrahymena* proteinase | Heat stable | 171 |

[a] Not endogenous proteinases.

a vacuolar proteinase of *N. crassa* was released by reverse pinocytosis had to be discounted when it was found that the proteinase was a constitutive enzyme, whereas the extracellular enzymes were controlled by nutrient levels (105). Proteinases have been found in the exudates on various fungal structures (55). These are liquid droplets enveloped in membranous material. A study of the formation of *A. niger* acid proteinase in response to sulfur starvation revealed that this extracellular enzyme was produced by a portion of hypha at least 40 μm from the growing tip (368).

The relationship between intracellular and extracellular fungal proteinases has received little attention. In *A. nidulans*, Cohen (47) has described three extracellular neutral-alkaline proteinases (α, γ, and ε) and one proteinase (β) that is strictly intracellular. The extracellular enzymes were also detected in mycelial extracts. An active precursor of γ, proteinase δ, was also detected in mycelial extracts and culture filtrates but was only converted to γ in stored extracts. In ammonium-repressed cultures, the only proteinases detectable were two precursor forms of the intracellular proteinase β. Ansari and Stevens (7) have recently described three intracellular proteinases from *A. nidulans*, proteinases A, B, and C, which probably correspond to proteinases β, α, and δ, respectively.

In *N. crassa*, two intracellular enzymes, identical to the extracellular alkaline and neutral proteinases, have been reported (74). Maximum levels of the intracellular enzymes were detected when rates of secretion of proteinase activity were maximal. A strictly intracellular serine proteinase has also been detected (74, 157).

Proteolytic enzymes bound to the cell wall of *Saccharomyces sake* (160), the mycelial surface of *Mucor hiemalis* (348), and membranes of *A. oryzae* (340) have been described. The membrane-bound acid proteinases of the latter species are intracellular and are present in rough and smooth microsomes but closely resemble the extracellular proteinases (340).

The difficulties associated with the preparation of fungal cell extracts by techniques which allow the isolation of intact organelles have limited the study of intracellular proteinase localization. In yeast, the major proteinases A and B, together with carboxypeptidase Y and the high molecular weight aminopeptidase, are in

vacuoles, organelles which are functionally related to lysosomes. Wiemken et al. (352) have estimated that for each of the four enzymes more than 90% of the total cellular activity is vacuolar. Other peptidases and the inhibitors of proteinases A and B and carboxypeptidase Y are cytosolic (see reference 361). The localization of carboxypeptidase S and the new proteolytic activities reported by Achstetter et al. (3) have not yet been established, but some may be membrane associated. Vacuolar proteinases have also been reported in *N. crassa* (105), and *C. albicans* (32). Page and Stock (248) fractionated a macroconidial extract of *Microsporum gypseum* on Ficoll and found three types of lysosome-like particles. Two of these contained proteinase activity, one with acid proteinase and the other with alkaline proteinase, the latter being associated with the spore coat. On germination, the alkaline proteinase became extracellular.

Mitochondrial proteinases have been reported in yeast (196, 237). The mitochondrial activity was different from the vacuolar proteinases, as it was not inhibited by antisera against proteinases A and B but was inactivated by leupeptin, which has no effect on the latter enzymes (134). Proteinase activity has been reported in mitochondrial preparations from *Schizosaccharomyces pombe* and *N. crassa* (203), and a mitochondrial enzyme responsible for cytochrome oxidase turnover has also been described in *N. crassa* (158).

Extracellular proteinase activity has been reported in the protozoan *T. pyriformis* (25, 26, 67, 321). Suprynowicz and Allewell (321) found that an enhanced rate of proteinase secretion occurred after transfer to nonnutritive medium and was paralleled by a decrease in intracellular activity. Blum (26) has shown that acid proteinase secretion is accompanied by the release of other lysosomal hydrolytic enzymes. Few other attempts to detect extracellular activity in protozoa have apparently been made. Proteinase activity has, however, been detected on the surface of *E. histolytica* trophozoites (87) and on the surface of *T. cruzi* cells at different developmental stages (264). The acellular slime mold *P. polycephalum* produces extracellular proteinases (75, 98). Rossomando et al. (278) have reported the release of proteinase activity from the cellular slime mold *D. discoideum* when myxamoebae are starved in buffer. The pattern of proteinases released under these conditions is similar to that of the intracellular enzymes (231).

Early observations on centrifuged amoebae had shown that their proteinases were localized within discrete organelles (114). Subcellular fractionation has since revealed that in many protozoa a large proportion of the proteinase activity is associated with lysosome-like vacuoles which contain other hydrolytic enzymes (73, 179, 184, 198, 310). Many of the proteolytic activities of the cellular slime mold *D. discoideum* are also recovered in a lysosomal fraction (232, 353), although the leucine aminopeptidase is not particulate. Proteinase I shares a common antigenic group with the lysosomal enzyme β-*N*-acetyl-glucosaminidase (96).

## THE ROLE OF PROTEINASES

### Methods of Assessment

Although the in vitro properties of a proteinase and a knowledge of its localization may provide some indication of its in vivo role, conclusions made solely on this basis must remain speculative. In addition, alterations to proteinase levels during physiological responses or developmental changes do not necessarily reflect causal relationships. Nevertheless, much of what we know about proteolysis and the proteinases of lower eucaryotes is based on such observations.

A more direct approach is dependent on the ability to manipulate proteinase activity in vivo. Many inhibitors are now available and have been used for in vivo studies. However, not all are necessarily specific for individual proteolytic enzymes, and there is often a danger that processes other than those involving proteinase activity might be affected (259, 277, 296). In this respect, the *Streptomyces* inhibitors such as pepstatin and S-PI, and antipain, leupeptin, and chymostatin are of particular value, since side effects have not been reported. However, unless it is possible to demonstrate unequivocally that only one particular proteinase is inhibited by the agent, conclusions must be limited.

A more precise means of manipulating proteinase activity in vivo is through the isolation of appropriate mutants. This necessitates the use of an organism amenable to selection procedures and suitable for genetic analysis. Since 1975, yeast mutants have been isolated which have low levels of or totally lack proteinase A (22, 200), proteinase B (357, 378), carboxypeptidase Y (132, 360), and carboxypeptidase S (359). Carboxypeptidase S was first detected in a carboxypeptidase Y-less mutant (362). A triple mutant lacking proteinase B, carboxypeptidase Y, and carboxypeptidase S activity was used to examine new yeast proteolytic enzymes (3), and a mutant with low levels of proteinases A and B and carboxypeptidase Y has been used for the detection of a new X-prolyl-dipeptidyl aminopeptidase (317). At least some of the mutations are probably in structural genes of the proteolytic enzymes (200, 379). A mutant with reduced proteinase A inhibitor has also been found (18).

In *Saccharomycopsis lipolytica*, mutants producing reduced levels of extracellular proteinase have been isolated. At least 16 genes are involved (303). Some mutations are regulatory, but the *xpr-32* mutation is probably in the structural gene *XPR2* for the alkaline proteinase (303). Extracellular proteinase mutants have also been isolated in *A. nidulans*: *xprC1* strains have lost the ability to produce extracellular proteinase (47), whereas *xprD1* strains have control mutations and produce proteinase in the presence of the repressor, ammonia (46). The *uvs-3* and *uvs-6* mutations of *N. crassa* have been found to result in the lack of proteinase (273). A number of mutations affecting cell lysis associated with protoplasmic incompatability in *P. anserina* have been isolated, some of which have altered proteinase activity. The *modC* strains have suppressed lytic proteinases (162), and *modD* strains have defects which can be suppressed by the presence of the *modC* mutation or by β-phenylpyruvic acid, a proteinase inhibitor (163).

Many attempts have been made to select fungal strains that produce higher levels of proteinase, particularly among the aspergilli. However, the aim has often been simply to improve enzyme yields and not to understand the control of proteinase synthesis or secretion.

The role of proteinases has also been assessed by examining the correlation between the proteinase activity of closely related organisms and a particular biological activity. This has been used in particular with pathogenic organisms (see below). However, comparisons have often been made by considering the proteinase levels found under culture conditions, which might not bear any relationship to those experienced during infection. Conclusions based on such comparisons are limited.

### Posttranslational Processing

Primary translation products are often larger than the final product, and proteolysis must be involved in the subsequent processing. Johnson and Brown (131) looked for proteolytic enzymes from *N. crassa* which might be involved in the removal of the $NH_2$-terminal methionine. The dipeptidases they detected were not thought likely to play such a role, however. More recently, the role of proteolytic enzymes in the cotranslational transport of secretory polypeptides across microsomal membranes and the posttranslational transport of polypeptides into organelles has become a major area of interest (153).

A proposal that yeast cytochrome *c* oxidase subunits IV to VIII are synthesized as a cytoplasmic polyprotein precursor subsequently processed in the mitochondrion (262) has since proved to be incorrect. Nevertheless, it is clear that there are larger precursors for each of subunits IV, V, and VI which must be processed proteolytically during or shortly after entry into the mitochondrion (63, 153). Recently, McAda (196) has reported the partial characterization of a mitochondrial proteinase believed to be involved in the processing of the precursor of yeast adenosine triphosphatase subunit 2. A thiol-dependent proteinase responsible for the processing of a precursor for the small subunit of ribulose 1,5-bisphosphate carboxylase has been reported in the alga *Chlamydomonas reinhardtii* (69).

The polypeptide portion of yeast carboxypeptidase Y is synthesized as a larger precursor (103). Maturation of the enzyme involves both proteolysis and glycosylation and has been demonstrated in vitro (213). Hemmings et al. (106) have described a mutation, *pep4-3*, that prevents precursor maturation for carboxypeptidase Y and at last four other vacuolar enzymes. Although proteinase B will catalyze the conversion of carboxypeptidase Y precursor to mature polypeptide in vitro (103), it cannot be responsible for the in vivo proteolytic processing (106).

Two examples of posttranslational processing have been reported in *Tetrahymena*. An enzyme in the postmicrosomal supernatant which could hydrolyze *N*-benzyl-L-tyrosine ethyl ester at pH 8 was believed to be responsible for the proteolytic cleavage of pellicular proteins (54). The inhibitor TPCK increased the level of precursor protein. Proteolytic processing of histone H3 in the chromatin of micronuclei has also been described (5). It involves the removal of the first six residues from the $NH_2$-terminus. The enzyme responsible has not been described.

### Protein Turnover

The turnover of cellular protein, first reported in animals by Schoenheimer (287), allows the cells to remove abnormal proteins and to adapt their complement of protein more rapidly to changing physiological needs. Most demonstrations of protein turnover in microorganisms have involved bacteria (256), and relatively few measurements have been made on lower eucaryotes. Most of these have concerned turnover during starvation and differentiation, and in very few have growing cells been examined. A temporal relationship between levels of protein turnover and proteolytic enzymes can be seen in starving and differentiating cells of a number of species. In yeasts, the levels of proteinase A and B increase during sporulation as protein turnover increases (23). When sporulation is inhibited with ammonium ions, there is a decreased

rate of protein turnover and decreased proteinase activity (64, 245). The vital role of the yeast proteinases in protein turnover can be assessed from the reduction of 30% and over 40% in protein degradation observed during sporulation of diploids lacking proteinase A (200) and proteinase B (358, 377), respectively. Similar observations have been made in starving mutant cells which lack proteinase B (358).

Increases in proteinase activity coincident with increased protein degradation have been observed in *Achlya bisexualis* (333) and *B. emersonii* (186). Antipain blocks sporulation in *B. emersonii* and decreases protein degradation, although the effect on sporulation is only apparent if the inhibitor is present during the earliest phases (62).

Protein turnover is also triggered by starvation in the slime molds and has been noted during differentiation of both *P. polycephalum* (350) and *P. flavicomum* (109). It also occurs during fruiting body formation in the cellular slime mold *D. discoideum* (322), but the level of proteinase activity, at least in the acid pH range, does not increase (353). Indeed, decreases in both the cathepsin D-like and cathepsin B-like activities have been reported (79, 80). Fong and Bonner (79) have shown that chloroquine blocks the development of *D. discoideum* and that this treatment leads to depleted amino acid pools. The effect could be reversed by the addition of amino acids. Similar results were obtained with TLCK, except that glutathione was also needed to reverse the effect. Since both agents inhibit the cathepsin B activity (80), it was suggested that proteolysis has a regulatory role during development. However, chloroquine inhibits other enzymes from *D. discoideum*, including proteinase E (a cathepsin D-like enzyme), leucine aminopeptidase, and some non-proteolytic hydrolases (236). The latter observation complicates the interpretation of any in vivo effects of chloroquine which are not necessarily due to inhibition of cathepsin B activity. Further consideration of the developmental role of proteinases is given below.

Mitochondrial protein turnover has been studied in yeast. Rapid turnover of mitochondrial translation products is believed to be due to the removal of faulty protein during mitochondrial biosynthesis (83). The enzyme believed to be responsible has been identified by Kal'nov et al. (134).

### Activation and Inactivation of Specific Proteins

Protein turnover measurements involve the total protein of a cell or organelle. However, proteolytic events are often more specific, resulting in the selective activation and inactivation of individual proteins.

The activation of zymogens by limited proteolysis is a well-known phenomenon, but there are few examples of its occurrence among the lower eucaryotes. However, chitin synthetase from many fungi can be isolated in an inactive form which can be activated by exogenous or endogenous proteinases (17, 32, 39, 343). However, the endogenous activators demonstrated in vitro are not necessarily the agents responsible for activation in vivo. The proposal that, in yeast, proteinase B is involved in the control of chitin synthetase activity and thus of chitin synthesis during growth (37) must be revised, since it has been found that mutants lacking proteinase B show no abnormalities in vegetative growth, septa formation, or cell division (358, 378).

A galactosyl transferase involved in osmoregulation of the phytoflagellate *O. malhamensis* also exists in an inactive form which can be activated by endogenous and exogenous proteinases (141).

Enzyme instability in cell extracts can often be attributed to proteolysis but may not necessarily have any physiological significance. However, in vivo enzyme inactivation in response to physiological or developmental changes is frequently observed in microorganisms (324). In yeast, a number of enzymes are inactivated when glucose is added to cultures growing on a poorer carbon source, a phenomenon known as catabolite inactivation (see references 354 and 361 for details). Because the decrease in enzyme activity is often paralleled by a loss of enzyme protein and can be inhibited in vivo by proteinase inhibitors, it was believed to be due to proteolysis. The major proteinases A and B each inactivate certain of the enzymes in vitro, and it has been suggested that these proteinases were responsible for inactivation in vivo. However, catabolite inactivation still occurs in mutants lacking proteinase A (200) and proteinase B (107, 214, 358, 376), showing that neither enzyme can be uniquely involved. Asparaginase II inactivation, which occurs during the transition to stationary phase, was not affected by a carboxypeptidase S mutation (254).

Results from Holzer's laboratory (115, 173, 337) now indicate that, at least for fructose 1,6-bisphosphatase, proteolysis represents only the second stage of the inactivation process, the first being a reversible phosphorylation which converts the enzyme to an inactive form. It is believed that the phosphorylated enzyme is then more susceptible to proteolysis, possibly because it is now recognized by receptors on the lysosome-like vacuoles.

When statically grown stationary-phase cultures of *T. pyriformis* are shaken, the specific activity of several peroxisomal enzymes de-

creases (179). During the same period, the level of intracellular neutral proteinase activity increases two- to threefold. Actinomycin D and cycloheximide prevent both the enzyme inactivation and the increase in proteinase activity. Levy and McConkey (177) observed that a purified preparation of proteinase was able to inactivate several commercial enzymes, including ones equivalent to those inactivated in vivo. However, the increased level of proteinase activity is not necessarily responsible for the in vivo inactivation, since Suprynowicz and Allewell (321) have noted that under other conditions, parallel decreases in peroxisomal enzyme activity and proteinase activity occur.

Selective enzyme inactivation (of phosphoglucomutase) has been observed during fruiting body formation in *Schizophyllum commune* (288). Schwalb (289) has described a proteolytic factor which can inactivate phosphoglucomutase in vitro. The factor could only be detected at the stage of the developmental cycle (stage II) coincident with enzyme inactivation. The inactivation factor is not specific for phosphoglucomutase or for *S. commune* enzymes, however.

In many reports on enzyme inactivation, decreases in enzyme activity have been described which occur during periods in which proteinase activity increases. However, the opposite of this has been observed during development of the cellular slime mold *D. discoideum*, that is, an increase in enzyme activity during a period in which proteinase activity decreases. The specific activity of UDP-glucose pyrophosphorylase increases at the aggregation stage of development, and at the same time the level of proteinase I activity decreases (97). Gustafson and Thon (97) have shown that UDP-glucose pyrophosphorylase is inactivated in vitro by purified preparations of proteinase I and have concluded that changes in enzyme and proteinase levels in the cells are not only temporally related but are also causally related. Further support for their proposal that UDP-glucose pyrophosphorylase levels are controlled through turnover catalyzed by proteinase I comes from earlier observations of De Toma et al. (66), who showed that precocious increases in UDP-glucose pyrophosphorylase activity could be induced by incubating cells with TLCK, an inhibitor of proteinase I, and that the activity in cell extracts was stabilized by the inhibitor. It seems likely, however, that the proteinase and the inactivated enzyme are in different cell compartments, since the esterase activity on α-*N*-carbobenzoxy-L-lysine *p*-nitrophenol ester, used to assay proteinase I (97), is recovered predominantly in the lysosomal fraction of myxamoebal extracts (232). It is difficult to envision how a lysosomal proteinase could specifically control the level of a cytosolic protein. The turnover of the enzyme is more likely to be influenced by cytosolic factors which may, for example, determine whether the enzyme can be taken up by lysosomes. It is also important to note that other workers (e.g., reference 81) believe that the increase in UDP-glucose pyrophosphorylase activity is more likely to be due to increased rates of synthesis and not to decreased rates of enzyme degradation. The role of proteolysis in controling enzyme levels during development has been discussed by Wright and Thomas (365) but without reference to specific proteinases.

Changes in the size of cyclic adenosine monophosphate binding proteins during *D. discoideum* development have also been attributed to proteolysis (61). Conversion of a higher molecular weight form (found at later stages of development) to a lower molecular weight form (found at earlier stages of development) can be achieved in vitro by mixing vegetative cell extracts with late developmental cell extracts: the conversion is inhibited by the proteinase inhibitor TLCK (S. Cooper, personal communication). The time at which the change in binding proteins occurs corresponds to the time at which proteinase I activity decreases (S. Cooper, personal communication). Again it would seem unlikely that a lysosomal proteinase would be able to control specifically proteolytic events involving cytosolic proteins, however.

### Proteinases and Nutrition

An obvious role for proteinases in organisms which utilize protein as a nutrient source is in the digestion of food. In fungi, this would involve breakdown outside the cells by extracellular enzymes, whereas in the phagotrophic protozoa and slime molds digestion almost certainly takes place within intracellular vacuoles. A role for the extracellular proteinase of *Tetrahymena* in protein utilization has been discounted, since a phagocytosis-deficient mutant could not utilize egg albumin (266). The level of extracellular proteinase of *E. gracilis* was increased by the addition of peptone to the medium (225) and was higher in a bleached mutant, suggesting that in this case the extracellular enzyme may have a role in heterotrophic growth (225). However, very little study has been made of how the proteinases of either protozoa or slime molds respond to changes in the nutrients, although the effects of starvation and developmental changes have been studied. In *T. pyriformis*, an increase in the level of intracellular proteinase activity has been noted after ingestion of yeast (271). The pattern of intracellular acid proteinases of *D. discoideum* is basically the same for cells grown on bacteria as it is for cells grown axenically (234). The proteinases can hydrolyze the

protein moeity of a partially purified lipoprotein complex from *Escherichia coli* (33).

Fungal proteinase levels do respond to changes in nutrients, and both induction and derepression of extracellular proteinases have been noted. Some of the more detailed studies have been those of Cohen on *A. nidulans* (46, 48) and other species of *Aspergillus* (51). Extracellular enzyme production occurs under conditions of nitrogen, carbon, or sulfur limitation but does not require the presence of protein. Similar observations have been made in less systematic studies of *Aspergillus* and other species (50, 126, 128, 145, 243, 300, 368) which have demonstrated repression of proteinase production by various nutrients. Reports that repression by glucose can be reversed by cyclic adenosine monophosphate have appeared (14, 145). In other fungal species, proteinases are not only subject to repression but must be induced by extracellular protein. This induction has been studied in most detail in *N. crassa* by Drucker and Cohen and their co-workers (52, 53) and by Hanson and Marzluf (100). Proteolytic activity is required before induction can occur (71). Interestingly, there is now evidence that in the *N. crassa* system different acid proteinases are produced under different conditions of derepression (182), although the same alkaline and neutral proteinases are induced regardless of whether it is nitrogen, carbon, or sulfur that is limiting (53, 100). Proteinase induction has also been reported in *M. miehei* (167), *Trichophyton rubrum* (201), *Microsporum canis*, (246), and *C. albicans* (268). The role of proteinases in the breakdown of more complex nutrients has received little attention except for pathogenic species (see below). However, Fermor and Wood (76) have recently reported an increase in the level of a neutral extracellular proteinase during growth of *Agaricus bisporus* on killed bacteria.

Direct evidence that peptidases are involved in nutrient utilization has recently been obtained in yeast. A double mutant lacking carboxypeptidases Y and S was unable to grow with *N*-carbobenzoxy-L-glutamyl-L-leucine as the sole nitrogen source (359).

### Proteinases and Development

Proteolysis might play a number of roles during morphogenesis and differentiation in microorganisms. Since development often occurs as a result of starvation, general protein turnover is essential for supplying amino acids for de novo protein synthesis (see above). Proteinase activity may also be needed for the selective inactivation of specific growth phase proteins not required during development and for the activation and modification of others that are required.

In fungal cultures, increases in proteinase activity are often associated with the end of the growth phase, and links with cytodifferentiation have been noted. Among the fungal systems in which development can be followed under controlled experimental conditions, increases in proteinase activity have been noted during sporulation in yeast (23, 146, 355), sporangia differentiation in *A. bisexualis* (333), sporulation in *B. emersonii* (186), cleistothecium differentiation in *A. nidulans* (375), fruiting body formation in both *S. commune* (34, 289) and *A. bisporus* (363), and carotenogenesis in *B. trispora* (88). The significance of this increase to developmental events has not been firmly established in all cases. Indeed, a large increase in *A. nidulans* intracellular proteinase activity does not always occur under conditions which allow cleistothecia development. Zonneveld (375) has concluded that the proteinase action can supply carbon and energy for sexual differentiation, but this is dependent on whether external glucose or glucan are also available and may be controlled by cyclic adenosine monophosphate levels. Mutants of *A. nidulans* lacking extracellular proteinase develop normally (47).

Sporulation in *B. emersonii* is blocked by antipain, an inhibitor of the alkaline proteinase. The effect is not reversible, but antipain is effective only during the first 60 min of sporulation, suggesting a critical role for the proteinase for a limited period of time during the initial phases of sporulation (62). Although sporulation in yeast proved insensitive to most inhibitors of proteinases tested (23), it is partially affected by mutations which result in a lack of proteinase A (200) and proteinase B (358). Carboxypeptidase activity may also be important, since triple mutants lacking carboxypeptidases S and Y and proteinase B have almost completely lost the ability to form ascospores (359). The proteolytic enzymes would be required to provide amino acids from preexisting protein. An additional role for proteolysis in yeast is the degradation of the tridecapeptide α factor pheromone (44). The factor is cleaved at the Leu(6)—Lys(7) bond by the target *a* cells to yield an inactive fragment. The surface-bound activity is present in mutants lacking proteinase A or B or carboxypeptidase Y and may be due to one of the new proteolytic enzymes described by Achstetter et al. (3).

In *Blakeslea trispora*, a neutral particulate proteinase may trigger carotenogenesis by removing an inhibitor. However, this has only been demonstrated in vitro (88).

In *P. anserina*, four proteinases appear to play a role in cell lysis associated with protoplasmic incompatability. The lytic enzymes, proteinases I, II, III, and IV, are not found in vegetative cells (161). Studies involving mutants, in which

the proteinases are either suppressed or elevated, or the administration of β-phenylpyruvic acid, which inhibits the acid proteinases III and IV, have also related the activity of these proteinases to protoperithecum formation, ascospore outgrowth, and renewed growth of stationary cells (164).

The formation of fruiting bodies in some species of basidiomycetes is enhanced by the aspartic proteinase inhibitor S-PI (329–331). In *Lentinus edodes*, S-PI administration decreased the level of an S-PI-insensitive extracellular proteinase while increasing that of a metalloproteinase (331). However, the mechanism by which this and the enhancement of fruiting body formation is achieved is not known.

The cellular slime mold *D. discoideum* has proved to be an excellent model system for developmental studies. During fruiting body formation, the specific activity of the intracellular acid proteinase activity remains unchanged (353), but there are decreases in the activity of individual cysteine proteinases (79, 80, 97, 232, 234). The cathepsin B-like activity decreases to a greater extent in prespore than in prestalk cells (80). Leucine aminopeptidase activity increases during development (77), as does that of enzymes responsible for the hydrolysis at pH 7.5 of the chromogenic substrates acetyl-Ala-Ala-Pro-Ala-*p*-nitroanilide, succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide, and succinyl-Ala-Ala-Pro-Leu-*p*-nitroanilide (232), all of which are nonlysosomal. One new form of acid proteinase appears just before fruiting body formation (234). Evidence for a specific developmental role for individual proteinases remains circumstantial. Fong and Bonner (79) have reported that development is inhibited by chloroquine, TLCK, antipain, and leupeptin, all inhibitors of the cathepsin B activity. Since the effects of chloroquine and TLCK could be reversed simply by the addition of amino acids, the results show that those enzymes which are inhibited by these agents are responsible only for supplying the cells with amino acids either for de novo protein synthesis or for energy. More subtle proteolytic events cannot be dependent on proteinases inhibited by TLCK or chloroquine, since such events would not be substituted by ed by the addition of amino acids to the medium. As discussed above, the specificity of chloroquine is also in doubt (236), and a more detailed analysis of the developmental effects of antipain and leupeptin would be useful. The suggestions that *D. discoideum* proteinase I controls developmentally regulated changes in UDP-glucose pyrophosphorylase and cyclic adenosine monophosphate binding sites are discussed above.

A developmental role for extracellular proteinase in *D. discoideum* has been suggested by Rossomando et al. (278), who proposed that the proteinase released by myxamoebae on starvation could be responsible for cell surface modification before aggregation. This seems unlikely in view of the finding that proteinase release is not energy dependent and varies with the degree of experimental perturbation to which the cells are exposed (231).

In *E. gracilis*, a PMSF-sensitive proteinase may have a role in supplying amino acids for chloroplast development, since light-dependent chloroplast formation is blocked by PMSF but is relieved by the addition of a nitrogen source, ammonium phosphate (374). It was proposed that light stimulates a proteinase-dependent breakdown of protein to supply the amino acids.

During germination processes, proteinase activity may be required for hydrolyzing spore or cyst coat proteins or for mobilizing storage proteins. However, little detailed study has been made in lower eucaryotes. In *H. culbertsoni*, excystment can be triggered by treatment with trypsin (138), and normal excystment is accompanied by the release of hydrolytic enzymes, including two proteinases (140). Proteinase release has also been observed during microcyst germination in the cellular slime mold *P. pallidum* (242). An interesting change in proteinase property has been reported to take place during *M. gypseum* macroconidia germination (249). There is an interconversion between an intracellular alkaline germination proteinase and an extracellular keratinase, a process that is triggered by phosphate.

### Proteinases and Pathogenesis

Many eucaryotic microorganisms are pathogenic, causing diseases in plants as well as in invertebrate and vertebrate animals. For the interaction between host and pathogen, proteinases may be needed by the latter to penetrate the host tissue or to utilize host proteins for nutrition.

There have been many demonstrations of proteolytic activity in cultures of fungi pathogenic to plants and also in infected plant material. However, few have provided direct evidence for a definite role for proteinases in infection. It may often be the case that proteinases detected in culture are actually needed for the saprophytic growth of a species in the soil and not for infection. However, a correlation between pathogenicity and proteinase activity has been reported for *Colletotrichum lagenarium* (257) and for *Sclerotinia sclerotiorum* and *Sclerotinia minor* (142). Ries and Albersheim (272) considered the possibility that the proteinase from *Colletotrichum lindemuthianum* might play a role in degrading the hydroxyproline-rich structural proteins of the cell wall, since it was secreted shortly after endopolygalacturonidase

activity. Such a role was considered to be unlikely, since the proteinase was not very effective at hydrolyzing the proteins of sycamore or tomato plants. Hislop et al. (112) have recently concluded that the acid proteinase from *Monilinia fructigena*, which they have purified, probably does not have a direct role in the host (apple fruits)-pathogen interaction but might be involved in nutrition.

Plant tissues contain proteinase inhibitors, some of which are active on fungal proteinases, including those of known pathogens (148, 210, 211). Peng and Black (255) have reported increased proteinase inhibitor activity in resistant tomato plants in response to infection by *Phytophthora infestans*. The response was different for compatible and incompatible races.

Swinburne (323) has made the interesting proposal that a fungal proteinase could itself elicit a protective mechanism within the host plant. Resistance of immature apples to rotting by the fungus *Nectria galligena* has been attributed to their ability to accumulate benzoic acid. Proteinase preparations from *N. galligena* and other fungal species can stimulate benzoic acid accumulation. Phytoalexin production was not stimulated when bean cotyledons were treated with *M. fructigena* acid proteinase, however (112).

A number of reports have appeared on the relationship between the proteinase activity of fungi pathogenic to insects and their pathogenicity. Sikura and Bevzenko (302) found that the virulence of *Beauveria bassiana* was not related to the activity of proteolytic or indeed lipolytic enzymes produced under deep culture conditions. In fact, Paris and Segretain (253) observed an apparent inverse correlation between the extracellular proteinase of *Beauveria tenella* and virulence on cockchafer larvae. Nevertheless, Samšiňaková et al. (284) have found that the amount of proteolytic and chitinolytic activity of both *B. bassiana* and *Paecilomyces farinosus* was in accord with their pathogenicity on the Colorado beetle. Kučera (154) has described a toxic proteinase produced by *B. bassiana* in culture, and cultivation medium of the same species has been shown to degrade the cuticle proteins of greater wax moth larvae (285). *Metarhizium anisopliae* also produces a high molecular weight fraction containing proteolytic enzymes which is toxic to larvae of the greater wax moth (155). Inhibition of one of the proteinases, P1, with PMSF decreased the toxicity threefold. Production of the proteinase activity by submerged cultures of *M. anisopliae* was highest when protein from the greater wax moth was used as the nitrogen source (156).

In the crayfish parasite *Aphanomyces astaci*, the proteinase activity remains the same during and after germination and in the initial growth of the hyphae. There is no release of extracellular proteinase during this period (304). It has been suggested that if the proteinase is required for penetration of the proteinaceous epicuticle of the host by the fungal germ tube, then the proteinase must be attached to the fungal cell wall. Subsequently, proteinase may weaken the cuticle invaded by mycelium, since proteinase release can take place on autolysis (305).

Before concluding a discussion of the invertebrates, it is important to record that not all fungal-invertebrate interactions involving proteinases are harmful to the latter. The proteinases of the gut of the fungus-growing ant, *Atta texana*, are almost certainly derived from the mycelial fluid of the fungus on which it feeds (31).

Many fungal species that are pathogenic to humans also possess proteolytic activity. For *C. albicans*, however, it is doubtful whether proteinase activity is always necessary for pathogenesis. In a study of denture stomatitis, Budtz-Jorgensen (35) noted that proteolysis was seen most frequently in *Candida* species (*C. albicans* and *C. tropicalis*) that were more pathogenic. Nevertheless, for 62 strains of *C. albicans*, there was no relationship between the severity of the inflammatory condition and proteolytic activity. Germaine et al. (85) found no evidence for proteolysis of salivary proteins in cultures of *C. albicans* grown at pH 7 or above. *Candida* proteinase was inactivated above pH 5 or 6; since the inactivation was PMSF sensitive, another proteinase may have been present (84). At lower pHs, proteinase production was inhibited by salivary proteins. Thus, it is likely that neither proteinase production nor activity can occur in the human oral cavity during infection.

Further evidence against a role for *C. albicans* proteinases in pathogenesis was presented by Saltarelli et al. (283), who found that, whereas four yeastlike strains produced extracellular proteinase activity, only one of the two more lethal mycelial forms was proteolytic under the same conditions. Nevertheless, since MacDonald and Odds (188, 189) have shown that antiserum against the acid proteinase could be useful for diagnosing candidosis, proteinase must be produced during some infections.

The role of proteinases in infections caused by dermatophytic fungi has also been studied. Various workers have reported keratinase, elastase, and collagenase activities elaborated by *Trichophyton* and *Microsporum* species (see reference 201). *Trichophyton mentagrophytes* var. *granulosum*, for example, produces both extracellular and cell-bound keratinases able to digest hair (370, 371). Antibodies against one of the cell-bound keratinases can be detected in the serum of dermatophyte-infected guinea pigs, although

the antibodies did not inhibit proteolytic activity (89). A relationship between proteolytic activity and inflammatory action has been demonstrated for *Trichophyton rubrum* and *T. mentagrophytes* (205).

There have been a number of suggestions that the proteinases of the protozoan parasites might play a role in the host-parasite interaction, although at present there is very little evidence to support this. However, a clearer understanding should be possible once more details of the proteolytic systems are known.

It is thought that in *T. cruzi* proteinase activity may be needed for penetration of the host cell (29). Support for this idea comes from the finding that antibodies against a purified cysteine proteinase bind to the cell surface of amastigotes (264). However, the authors report a cell surface location for the proteinase in other developmental forms, and the proteinase may alternatively be involved in the mechanism of escape from the immune system (264).

In *L. mexicana mexicana*, qualitative and quantitative differences between the proteinases of the two developmental forms have been demonstrated (58, 233). Coombs (58, 59) has suggested that the higher proteinase activity in the intracellular amastigote form may be related to the need to survive within the hostile environment of the host cell lysosomes. This could be achieved through the release of amino acids, which, on further metabolism, would yield ammonia and amines. These could elevate the lysosomal pH and thus reduce the activity of potentially harmful lysosomal hydrolases. It has recently been found that antipain, an inhibitor of the *L. mexicana mexicana* proteinases (58, 233), inhibits the growth of promastigotes and transformation from the amastigote to the promastigote form (59). It also inhibits the growth of amastigotes in macrophages (G. H. Coombs, personal communication).

The idea that the tissue lesions associated with invasive amoebiasis caused by *E. histolytica* might be caused by hydrolytic enzymes, including proteinases, has been suggested by many workers. Recently, Lushbaugh et al. (187) have provided evidence in support of a direct relationship between a cytotoxin from *E. histolytica*, whose concentration correlates with strain virulence, and proteinase activity, since cytotoxin activity can be inhibited by α-1 antiprotease and α-2 macroglobulin. A purified preparation of the neutral cysteine proteinase was completely inhibited by human and rabbit sera, and McLaughlin and Faubert (197) had suggested that the inhibition was likely to be due to the α-2 macroglobulin fraction. A plasma membrane location for *E. histolytica* proteinase has been reported (87).

During malarial infection, proteolysis of hemoglobin probably supplies the intraerythrocytic form with all of its amino acids. Although this idea was based initially on indirect observations, it was supported by the finding that parasites grown in erythrocytes containing radioactively labeled hemoglobin possessed labeled protein (298). As discussed earlier, analysis of the proteinases responsible remains problematical because of the presence of erythrocyte enzymes in parasite preparations.

Mahoney and Eaton (190) have recently reported that a chloroquine-resistant strain of *P. berghei* contains higher proteinase activity than that of a normal chloroquine-sensitive strain. Chloroquine is a widely used antimalarial agent, and the appearance of resistant strains is of considerable concern. During infection of erythrocytes, a malarial pigment (hemozoin) forms which may be responsible for binding chloroquine and trapping the drug within the cells. Some workers believe that the pigment forms as a result of incomplete hemoglobin breakdown, and Mahoney and Eaton (190) suggested that the higher proteinase activity of the resistant strain prevents malarial pigment formation and consequently chloroquine accumulation. However, it has also been reported that the hemozoin does not contain protein and probably consists entirely of hematin released by autooxidation of hemoglobin (297). If this is so, it is difficult to envision how an alteration to the level of proteinase activity leads to a difference in hemozoin accumulation. Further analysis of this interesting and important problem is required. Although there have been suggestions that *Plasmodium* proteinases might be good targets for drugs, with the exception of one early report (212), there is no evidence that antimalarials directly inhibit proteinase activity. They have been reported to inhibit aminopeptidase activity of two rodent malarial species (42) and aminopeptidases of *B. hylomysci* (4) and *E. nieschulzi* (43). It is not known how this relates to their antimalarial action, however.

Proteinases may have a role in the invasion of host cells by *Plasmodium* merozoites. It has recently been shown that in vitro invasion of monkey cells by merozoites of *P. knowlesi* can be inhibited by proteinase inhibitors, particularly chymostatin and leupeptin (13). Merozoite proteinases which may be responsible have been detected.

A relationship between proteinase content and pathophysiological effect has been reported for the cattle parasite *B. bovis* (367). A difference in the proteinase activity (determined as esterase activity on *N*-α-*p*-tosyl-L-arginine methyl ester) has been found for virulent and avirulent strains. Proteinase preparations from

*B. bovis* induce kinin formation in vivo and in vitro, and only the virulent strain increased kinin levels in infected animals.

## APPLIED ASPECTS

Proteinases and peptidases have proved to be valuable reagents in laboratory, clinical, and industrial processes. They also play an essential role in a number of food processes involving microorganisms. Although it is not possible to describe all these applied aspects in detail, a brief summary is provided below.

### Reagents

The purified fungal proteinases tend to have too broad a specificity to be useful for protein sequence analysis. However, the metalloproteinase of *A. mellea* has a specificity for peptide bonds involving the amino group of lysine and has proved useful for limited cleavage of proteins (301). It also hydrolyzes bonds adjacent to formylated lysine (16) and modified cysteine (2-aminoethylcysteine) residues (70). Carboxypeptidase Y from yeast is a useful reagent for determining carboxyl sequences of peptides (104). It may also prove to be of value in peptide synthesis (279, 351).

Proteinase K, the serine proteinase from *T. album*, is often used during the preparation of nucleic acids to remove proteins and inactivate nucleases (see reference 72). A method has recently been described which allows selective removal of ribonuclease activity from commercial preparations of deoxyribonuclease activity with proteinase K (341).

### Clinical Uses

Ever since it was found that extracts of *A. oryzae* and *Aspergillus flavus* possessed fibrinolytic activity (309), many hundreds of fungal isolates have been screened for enzymes which might be useful as thrombolytic agents. There is a particular interest at present in the Soviet Union, where workers are attempting to adapt proteinase preparations to make them more effective. One serine proteinase preparation from *Aspergillus terricola*, terrilytin, has been coupled to dextran (183) and to antibodies against fibrin (27), both of which procedures decrease its affinity for serum proteinase inhibitors; the latter procedure also increases its fibrinolytic activity.

The use of aminopeptidase from *Aspergillus japonica* as a digestive aid has been suggested (319).

An inducible, extracellular proteinase from *C. albicans* has been tested as a diagnostic antigen for candidosis (188, 189).

### Food Industry

A number of traditional fermentation processes in the food industry involve the breakdown of protein by fungal enzymes. In many cases, the proteinases of the organisms involved have been studied, but the results have not yet been related to the food process itself (e.g., references 121 and 349). An exception to this is the role of proteolysis in soy sauce production, one of the many Oriental food processes involving fermentation by molds. The process was reviewed by Yong and Wood (369). Studies on *A. oryzae* (219) and *A. sojae* (220), both used in Japanese soy sauce production, have shown that the release of amino acids from soy bean protein results from the concerted action of many of the complex of proteolytic enzymes, both endo- and exopeptidases, produced by these organisms in koji culture. A study has recently been initiated on the proteinases of *A. flavus* var. *columnaris* used for the manufacture of soy sauce in Thailand (125).

Preparations of *A. oryzae* proteinase have been shown to be suitable for reducing the time necessary for the preparation of Philippino fermented hydrolyzed fish products (6).

Cheese ripening may also depend on the activity of fungal proteinases, which, together with lipases, contribute to the development of texture and flavor (144). Studies on the proteinases of *P. roqueforti* and *P. caseicolum* indicate that these enzymes play a fundamental role in the proteolysis induced by the molds during ripening (91). The use of microbial enzymes, including fungal proteinase preparations, to accelerate cheese ripening has also been investigated (306).

One of the most important uses of fungal proteinases in the food industry has been as rennin substitutes (313). Many fungal acid proteinases have milk-clotting activity, but only a few are useful in cheese making. Too high a proteolytic activity results in extensive breakdown of casein protein after clotting has occurred. The proteinases of three organisms, *Mucor pusillus* var. Lindt, *M. miehei*, and *Endothia parasitica*, are now widely used, sometimes blended with rennin. Other substitutes are still being investigated, including, recently, proteinases of *Rhizopus oligosporus* (227) and *Byssochlamys fulva* (320).

*A. oryzae* proteinase is widely used in the baking industry to help control bread texture and gain dough uniformity (15). The possibility of using a purified proteinase from *S. carlsbergensis* has recently been suggested (364).

More details of some of the industrial aspects of microbial proteinases may be found in a recent review by Aunstrup (9).

## CONCLUDING REMARKS

Although the importance of microbial proteinases in industry and medicine has been and will remain an important influence on the choice of organisms and enzymes investigated, it has also become apparent that a study of the proteinases of lower eucaryotes can provide an insight into the role of proteinases in general. As fractionation and assay procedures have improved, so a complexity has been revealed in some of the proteolytic systems closer to that anticipated from the range of biological events which might involve proteolysis. It has recently been reported, for example, that 25 different proteolytic enzymes can be detected in *N. crassa* (307) and that the proteolytic system of yeast comprises more than just the well-characterized vacuolar proteinases and peptidases (3). Indeed, the study of the yeast system has illustrated many of the benefits of using eucaryotic microorganisms for analyzing proteinase function. Because it has been possible to isolate proteinase mutants, many of the proposals for proteinase function in vivo can be examined more critically than had previously been possible. As recently emphasized in an article by Wolf (356), some proposals made on the basis of in vitro proteinase action have been shown to be incorrect as a result of examining mutant strains. Hopefully, the type of detailed study being undertaken in yeast can be extended to other organisms, for example, *A. nidulans*, *N. crassa*, and *D. discoideum*, in which additional functions for proteinases are apparent, although the list of organisms is at present limited by the lack of suitable systems for genetic analysis. This is unfortunately true for the protozoa, for which a combined biochemical and genetic approach would prove more difficult. Nevertheless, the study of proteinases in these organisms should still prove rewarding if only because of the possible role of proteinases in the pathogenesis of the parasitic species. There is certainly no reason to doubt that the study of the proteinases of many groups of eucaryotic microorganisms will continue to provide valuable information on the role of these important enzymes in all living organisms.

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# The mechanism of cancer-mediated conversion of plasminogen to the angiogenesis inhibitor angiostatin

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**ABSTRACT** **Angiostatin, a potent naturally occurring inhibitor of angiogenesis and growth of tumor metastases, is generated by cancer-mediated proteolysis of plasminogen. Human prostate carcinoma cells (PC-3) release enzymatic activity that converts plasminogen to angiostatin. We have now identified two components released by PC-3 cells, urokinase (uPA) and free sulfhydryl donors (FSDs), that are sufficient for angiostatin generation. Furthermore, in a defined cell-free system, plasminogen activators [uPA, tissue-type plasminogen activator (tPA), or streptokinase], in combination with one of a series of FSDs (*N*-acetyl-L-cysteine, D-penicillamine, captopril, L-cysteine, or reduced glutathione] generate angiostatin from plasminogen. An essential role of plasmin catalytic activity for angiostatin generation was identified by using recombinant mutant plasminogens as substrates. The wild-type recombinant plasminogen was converted to angiostatin in the setting of uPA/FSD; however, a plasminogen activation site mutant and a catalytically inactive mutant failed to generate angiostatin. Cell-free derived angiostatin inhibited angiogenesis *in vitro* and *in vivo* and suppressed the growth of Lewis lung carcinoma metastases. These findings define a direct mechanism for cancer-cell-mediated angiostatin generation and permit large-scale production of bioactive angiostatin for investigation and potential therapeutic application.**

Because tumor growth and metastases are dependent upon angiogenesis (1–3), the identification of agents that inhibit angiogenesis now represents a potential therapeutic approach for the control of cancer (4–7). Angiostatin, consisting of the first four of five kringle domains of plasminogen (8), is one of a number of angiogenesis inhibitors that are internal fragments of larger nonangiogenic precursor proteins (8–14); however, the mechanisms by which these fragments are generated *in vivo* remains unknown. Although the activity sufficient to cleave plasminogen to angiostatin is present in tumor-bearing animals and serum-free conditioned medium (SFCM) of human prostate carcinoma cells (9), the cancer-dependent mechanism of angiostatin generation has remained unknown. Recently, macrophage-derived metalloelastase was shown to produce angiostatin from plasminogen and may contribute to angiostatin generation in the murine Lewis lung carcinoma model (15). We now describe the enzymatic mechanism for the direct generation of human angiostatin from plasminogen by human prostate cancer cells and demonstrate the generation of bioactive angiostatin from human plasminogen in a defined cell-free system.

## MATERIALS AND METHODS

**Angiostatin Generation.** Angiostatin was generated from PC-3 cell SFCM as described (9). To generate angiostatin in a cell-free system, human plasminogen (0.2 μM) was incubated with 0.2 nM recombinant human urokinase (uPA; Abbott), 1.0 nM recombinant human two-chain tissue-type plasminogen activator (tPA; a gift from Henry Berger, Glaxo–Wellcome), or 8.0 nM streptokinase (Sigma) and with 100 μM *N*-acetyl-L-cysteine (NAC), D-penicillamine, captopril, L-cysteine, or reduced glutathione (Sigma) at 37°C overnight. To confirm the requirement for plasmin catalytic activity, recombinant plasminogens (16) (0.2 μM) were added to 100-μl aliquots of 50 mM Tris, pH 9.0/20 mM NaCl/0.2 nM human recombinant uPA (Abbott)/100 μM NAC (Sigma) and incubated at 37°C overnight. The angiostatin product was examined by Western blot as described (9).

**Protein Purification.** SFCM was applied to Reactive Red 120-agarose (Sigma) equilibrated with 50 mM Tris·HCl, pH 7.5/140 mM NaCl (TBS), and proteins were eluted with 1.0 M KCl. The eluate was dialyzed against TBS by using a 6- to 8-kDa cutoff membrane. Human plasminogen (0.2 μM) was incubated in 100-μl aliquots of SFCM, Reactive Red 120-agarose flow-through, dialyzed eluate, or combined flow-through and eluate at 37°C for 18 h. For anion-exchange chromatography, SFCM was diluted 1:5 in 50 mM Tris (pH 10.0) and applied to a High Q anion-exchange resin (Bio-Rad) and a linear gradient (50–300 mM NaCl/50 mM Tris, pH 10.0) was used for elution. The protein content of each fraction was estimated by measuring the absorbance at 280 nm and fractions were analyzed for angiostatin-generating activity. For isoelectric focusing, the SFCM was concentrated 10-fold by ultrafiltration (Amicon) using a molecular mass cutoff of 10 kDa and diluted 1:5 with sterile water to lower the NaCl concentration to 20 mM, and ampholyte carriers (pH 3.5–9.5, Bio-Rad) were added. The sample was then fractionated in a Rotofor Cell (Bio-Rad) that stabilized the proteins into 20 focused zones from pH 3.0 to 10.0. Each fraction was analyzed for pH and angiostatin-generating activity.

**Cofactor Detection.** Human plasminogen (0.2 μM) was added to 100-μl aliquots of the dialyzed Reactive Red 120

Abbreviations: uPA, urokinase-type plasminogen activator; tPA, tissue-type plasminogen activator; NAC, *N*-acetyl-L-cysteine; bFGF, basic fibroblast growth factor; FSD, free sulfhydryl donor; SFCM, serum-free conditioned medium; $AS_{CF}$, angiostatin produced in the cell-free system.

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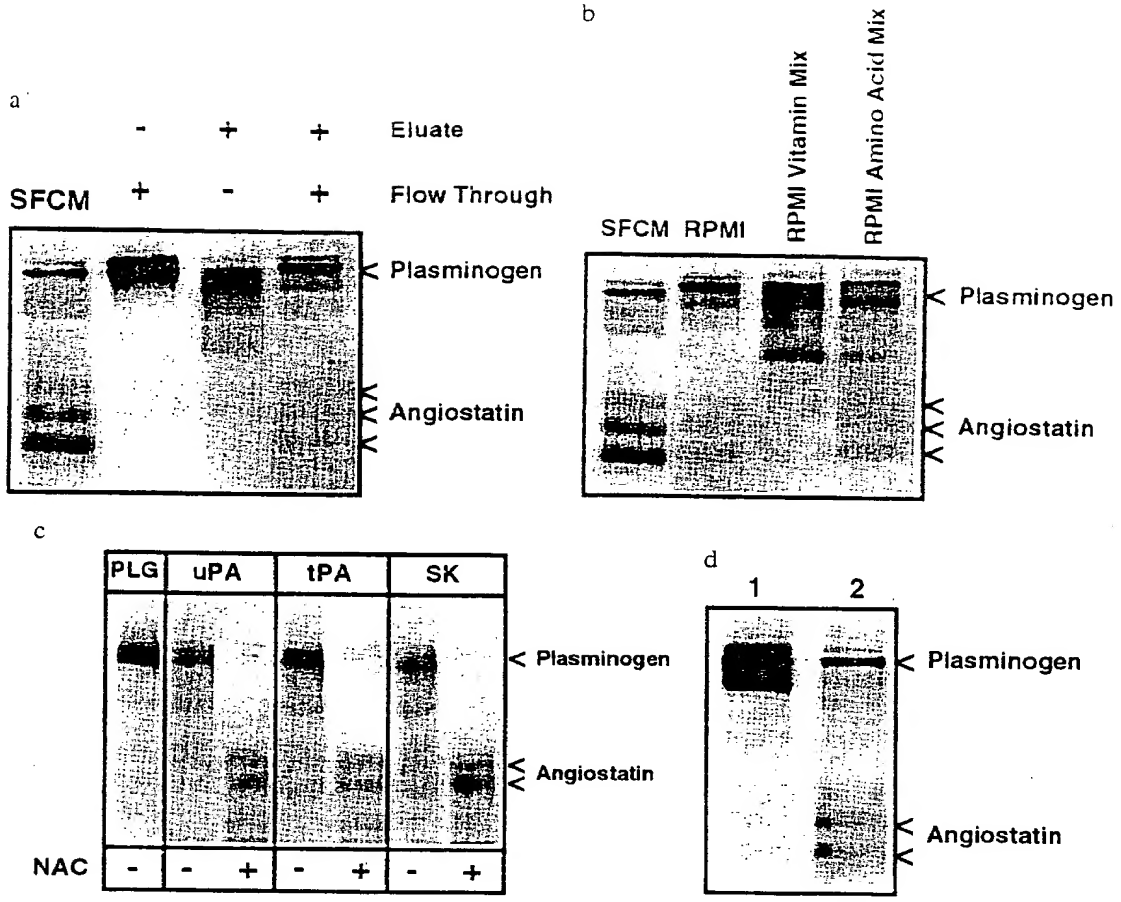


FIG. 1. Western blot analysis of angiostatin generation. (*a*) The angiostatin-generating activity of PC-3 SFCM required two distinct fractions from Reactive Red 120-agarose chromatography. Angiostatin was generated when plasminogen was incubated with SFCM. No angiostatin generation was detected by incubation of plasminogen with the Reactive Red 120-agarose flow-through or 1.0 M KCl eluate dialyzed against TBS. When the flow-through and dialyzed eluate were combined, however, angiostatin generation was restored. (*b*) A cofactor for angiostatin generation was present in unconditioned RPMI 1640 medium and its amino acid mixture. Plasminogen incubated with PC-3 SFCM generates angiostatin. Plasminogen incubated with the dialyzed Reactive Red 120-agarose eluate supplemented with unconditioned RPMI 1640 medium also generates angiostatin. The cofactor activity of RPMI 1640 medium, necessary for angiostatin generation, was not present in the RPMI 1640 vitamin mixture but was present in the RPMI 1640 amino acid mixture. (*c*) Angiostatin was generated in a cell-free system consisting of human 0.2 μM plasminogen (PLG), uPA, tPA, or streptokinase and the sulfhydryl donor *N*-acetyl-L-cysteine (NAC). Human plasminogen incubated with uPA (0.2 nM), tPA (1.0 nM), or streptokinase (8.0 nM) generated angiostatin only in the presence of NAC (100 μM). (*d*) Human PC-3 prostate carcinoma cells, cultured for 24 h in the sulfhydryl-depleted RPMI 1640 medium, secreted sufficient FSDs and uPA to generate angiostatin from plasminogen. Human plasminogen was incubated with sulfhydryl-depleted RPMI 1640 medium and uPA (lane 1) or with identical sulfhydryl-depleted RPMI 1640 medium conditioned by human PC-3 prostate carcinoma cells (lane 2).

agarose eluate supplemented with components in RPMI 1640 medium: salts, phenol red, a vitamin mixture, or an amino acid mixture (GIBCO/BRL). To define the cofactor necessary for angiostatin generation, the dialyzed eluate was incubated with individual components of RPMI 1640 medium, and samples were tested for angiostatin-generating activity by Western blot.

**Plasminogen Activator Detection.** Fractions from the anion-exchange and Reactive Red 120-agarose eluates were examined with a coupled assay that measures plasminogen activation by monitoring the amidolytic activity of generated plasmin. Briefly, the eluates were dialyzed against TBS and incubated with plasminogen (0.3 μM) and the plasmin substrate D-Val-Leu-Lys-*p*-nitroanilide (0.3 mM; Sigma) at 37°C. Substrate cleavage was determined by monitoring the absorbance at 405 nm using a kinetic plate reader (Molecular Devices).

**Plasmin Generation.** Human plasminogen (0.2 μM) in 100-μl aliquots of 50 mM Tris, pH 9.5/20 mM NaCl was incubated with 10 μl of uPA-Sepharose (Calbiochem) for 2 h at 37°C. After incubation, the sample was centrifuged to sediment the uPA-Sepharose, and the supernatant containing plasmin was collected. The complete conversion of plasminogen to plasmin was confirmed by analysis of the supernatant on reduced Coomassie-stained polyacrylamide gels. Plasmin was then incubated for 18 h with 100 μM NAC, and samples were analyzed for the presence of angiostatin.

**Bioactivity of Angiostatin.** The angiostatin, generated in a cell-free system, was purified by affinity chromatography on lysine-Sepharose (Pharmacia Biotech) and examined on Western blots as described in Gately *et al.* (9). Endothelial cell migration assays were performed in a modified Boyden chamber with bovine adrenal capillary endothelial cells (a gift from J. Folkman) as described (17). The mouse corneal angiogenesis assays were performed as described (18).

The Lewis lung carcinoma metastasis model was performed as described by O'Reilly *et al.* (8). In brief, $1 \times 10^6$ low-metastatic Lewis lung carcinoma cells were inoculated subcutaneously into C57BL6/J mice (The Jackson Laboratory). When tumors reached approximately 1200–1800 mg in size (12–14 days after implantation), animals were randomly divided into one of three treatment conditions: For the positive control group, mice were left with tumors intact ($n = 10$); for

the remaining animals, tumors were surgically resected. Tumor-resected mice received either cell-free-derived angiostatin, (0.15 mg, twice daily, subcutaneously) beginning on day 2 after surgery ($n = 6$) or, for negative control, received twice daily subcutaneous injections of phosphate-buffered saline ($n = 6$). Mice were sacrificed on days 25–27, and lung mass was measured to quantitate the growth of metastatic lung tumors.

## RESULTS

**Purification of the Factors Responsible for the Production of Angiostatin.** A significant loss of angiostatin-generating activity from human PC-3 prostate carcinoma cell SFCM (9) was observed after dialysis using 6- to 8-kDa molecular mass cutoff membranes, suggesting that a low molecular weight cofactor was required. Fractionation of SFCM on Reactive Red 120-agarose indicated that complementary components were required for angiostatin generation. The flow-through and the dialyzed eluate alone failed to generate angiostatin; however, combination of the flow-through and the eluate fraction restored angiostatin-generating activity (Fig. 1*a*). The flow-through component was stable to boiling, suggesting that this factor was not likely to be a protein. In contrast, the eluate component was thermolabile and was retained after dialysis consistent with the eluate containing a protein(s).

**The Flow-Through Component Necessary for Angiostatin Generation Was Identified as a Free Sulfhydryl Donor (FSD).** Addition of nonconditioned RPMI 1640 medium to the Reactive Red 120-agarose eluate resulted in the generation of angiostatin (Fig. 1*b*), indicating a component of RPMI 1640 medium could serve as a cofactor. Individual constituents of nonconditioned RPMI 1640 medium were then incubated with the dialyzed eluate. The amino acid mixture could complement the eluate for angiostatin generation (Fig. 1*b*), and testing of individual amino acids at concentrations present in RPMI 1640 medium indicated that L-cysteine is the only amino acid capable of complementing the eluate. The RPMI vitamin mixture (Fig. 1*b*) and other RPMI constituents could not serve as a cofactor. Because L-cysteine is a FSD, reduced glutathione (100 $\mu$M) and NAC (100 $\mu$M) were evaluated and also found to effectively complement the eluate for angiostatin generation.

**The Protein in the Elution Necessary for Angiostatin Generation Is a Plasminogen Activator.** Fractionation of SFCM using isoelectric focusing indicated that angiostatin generation was associated with an isoelectric point of approximately 9.2, similar to uPA (19). Furthermore, anion-exchange chromatography of SFCM resulted in the copurification of the angiostatin-generating activity with uPA, and the Reactive Red 120-agarose eluate contained plasminogen activator activity. The inability to separate uPA from angiostatin-generating activity suggested a role for uPA in angiostatin generation. The observation that plasmin was also converted to angiostatin by PC-3 SFCM (9) suggested that prior conversion of plasminogen to plasmin would not be inhibitory for angiostatin generation. Human plasminogen was therefore incubated with catalytic amounts of uPA, tPA, and streptokinase with and without NAC (100 $\mu$M; Fig. 1*c*). These data demonstrate that a plasminogen activator and NAC were sufficient for the complete conversion of plasminogen to angiostatin. Additional experiments indicated that other FSDs (100 $\mu$M L-cysteine, 100 $\mu$M reduced glutathione, 100 $\mu$M D-penicillamine, or 100 $\mu$M captopril) could substitute for NAC for the production of angiostatin. Incubation of plasminogen with uPA and a nonsulfhydryl reducing agent, dexrazoxane (Zinecard, Pharmacia), did not generate angiostatin, demonstrating the specific requirement for a FSD. These data indicate that incubation of human plasminogen with a plasminogen activator and a FSD is sufficient for conversion to angiostatin.

**Prostate Carcinoma Cells Release FSDs *in Vitro*.** Because RPMI 1640 medium contains FSDs in the form of L-cysteine and glutathione, to determine whether PC-3 cells release sufficient FSD to convert plasminogen/plasmin to angiostatin, PC-3 cells were cultured for 24 h in defined RPMI 1640 medium lacking reduced glutathione, L-cysteine, and L-methionine. This PC-3 SFCM was found to efficiently catalyze the conversion of plasminogen to angiostatin, indicating the cells release sufficient FSD and uPA for angiostatin generation (Fig. 1*d*).

**Plasmin as a Substrate for Angiostatin Generation.** Purified human plasmin, in the absence of uPA or other plasminogen activators, is converted to angiostatin in the presence of a FSD, suggesting a direct effect of the sulfhydryl donor on plasmin enzymatic activity or substrate specificity (Fig. 2*a*). To confirm a role for plasminogen conversion to plasmin and the catalytic role of plasmin in angiostatin generation, recombinant plasminogens (16) were evaluated as substrates for angiostatin generation. The *R561A* plasminogen activation site mutant is not susceptible to cleavage by plasminogen activators, whereas the *D646E* mutant yields a catalytically inactive plasmin due to a substitution of an essential amino acid in the serine proteinase catalytic domain. Both plasma-derived plasminogen and the wild-type recombinant plasminogen were converted to angiostatin when incubated with uPA and 100 $\mu$M NAC (Fig.

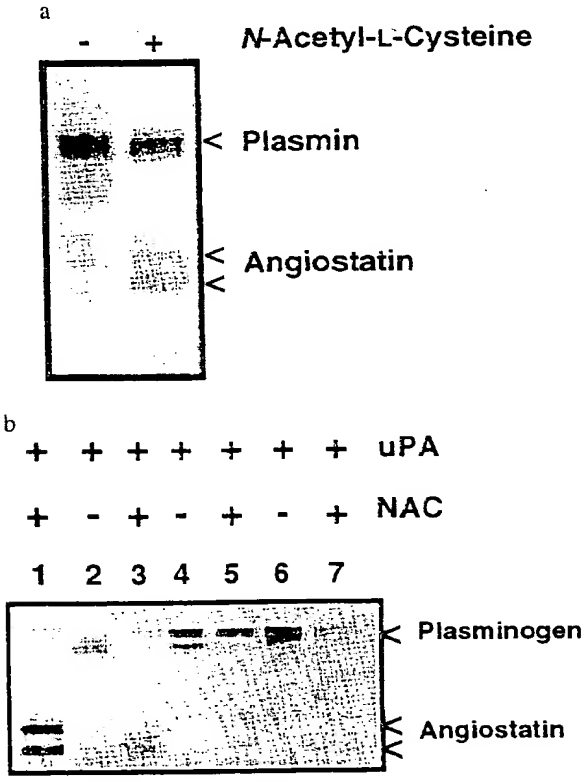


FIG. 2. (*a*) Plasmin is converted to angiostatin in the presence of a FSD. Human plasminogen was converted to plasmin by incubation with uPA-Sepharose. Plasmin was only converted to angiostatin in the presence of 100 $\mu$M NAC. (*b*) Plasmin generation and catalytic activity is essential for angiostatin generation. Plasma-derived human plasminogen (0.2 $\mu$M), incubated with uPA (0.2 nM) and NAC (NAC) generates angiostatin (lane 1). The recombinant wild-type plasminogen (lanes 2 and 3) is also converted to angiostatin by the addition of uPA and NAC. The *R561A* activation site mutant (lanes 4 and 5), not susceptible to activation by plasminogen activators, failed to generate angiostatin when incubated with uPA and NAC. The *D646E* catalytically inactive mutant (lanes 6 and 7) also failed to generate angiostatin, demonstrating the requirement for plasmin catalytic activity.

2*b*). However, the *R561A* mutant was not cleaved to plasmin or angiostatin under these conditions, providing further evidence that plasmin is an essential intermediate in angiostatin generation. The *D646E* mutant was converted to two-chain plasmin but angiostatin was not generated, demonstrating that plasmin catalytic activity is necessary for angiostatin generation (Fig. 2*b*).

**Bioactivity of Affinity-Purified Cell-Free-Derived Angiostatin.** The affinity-purified angiostatin produced in the cell-free system ($AS_{CF}$) was biologically active, suppressing basic fibroblast growth factor (bFGF)-induced endothelial cell proliferation with an $ED_{50}$ of approximately 15 μg/ml, similar to the PC-3-derived angiostatin (9). Inhibition of bFGF-induced endothelial cell migration *in vitro* by the cell-free angiostatin was comparable to the PC-3-derived (9) and elastase-generated angiostatin (generously provided by Michael O'Reilly, Harvard Medical School), with an observed $ED_{50}$ of 0.33 μg/ml (Fig. 3). As shown in Table 1, the cell-free angiostatin inhibited bFGF-induced angiogenesis in the mouse cornea as was shown for the PC-3-derived angiostatin (9).

Administration of the cell-free-produced angiostatin to mice significantly inhibited the growth of Lewis lung carcinoma metastases (Fig. 4). Surgical resection of primary subcutaneous Lewis lung tumors in mice resulted in numerous macroscopic metastases and a 71% increase in lung mass compared with animals in which the primary tumors were not resected (Fig. 4). By contrast, administration of angiostatin produced in a cell-free system suppressed the increase in lung weight to a comparable level as observed in animals with the primary tumor intact, and only microscopic metastases were observed. These data not only support the model that primary tumors can suppress the growth of metastases by the generation of an inhibitor of angiogenesis, angiostatin, but also confirm the biological activity of the cell-free-produced angiostatin.

## DISCUSSION

The results presented demonstrate the mechanism by which human prostate carcinoma cells convert plasminogen to the angiogenesis inhibitor angiostatin. Plasminogen is first converted to the two-chain serine proteinase plasmin, by uPA, and in the presence of a FSD, plasmin serves as both the substrate and enzyme for the generation of angiostatin (Fig. 5). This pathway was confirmed by the ability to convert plasminogen to angiostatin in a cell-free system using one of three available plasminogen activators and one of a series of physiological or pharmacological FSDs. Furthermore, the angiostatin generated in the cell-free system was shown to be bioactive, demonstrating antiangiogenic activity *in vitro* and *in vivo* and suppressing the growth of lung metastases in the mouse Lewis lung carcinoma model.

The local or systemic availability of FSDs may be an important regulatory point in the angiogenic cascade in physiologic and pathologic settings. The role of the FSD is not yet known but could be involved in modification of the conformation of plasmin, altering enzymatic activity or allowing plasmin to be cleaved at previously unrecognized sites. The observation that a FSD is required for angiostatin generation suggests that the reported antiangiogenic properties of pharmacologic sulfhydryl donors such as D-penicillamine and captopril (20–24) may be due to their ability to promote the conversion plasmin, a normally proangiogenic proteinase (25), to the angiogenic inhibitor angiostatin. The potential loss of plasmin catalytic activity that would result from plasmin conversion to angiostatin (Fig. 5) may contribute to reduced

Table 1. *In vivo* inhibitory activity of cell-free produced angiostatin

| Compound tested | No. positive corneas/ total no. implanted |
|---|---|
| bFGF (50 ng per pellet) | 4/4 |
| Angiostatin (200 ng per pellet) | 0/4 |
| bFGF + angiostatin | 0/4 |

Pellets were formulated with the indicated compounds and implanted into the corneas of mice, and neovascularization was assessed by slit-lamp microscopy 5 days later. Vigorous growth of vessels into the normally avascular cornea was scored as a positive response.

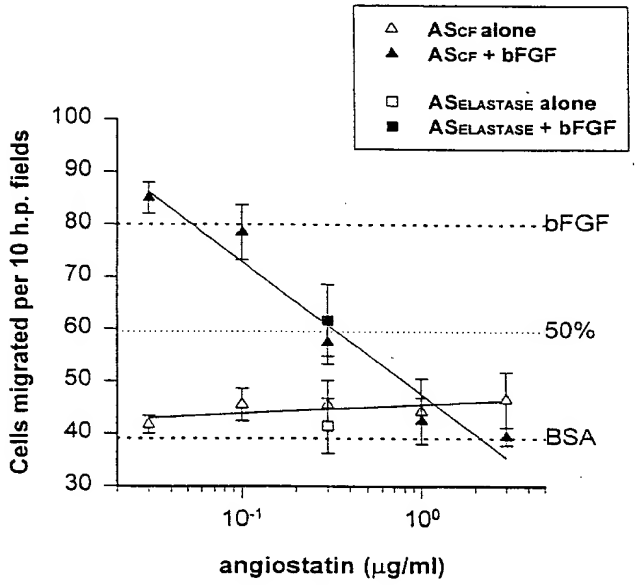


FIG. 3. Inhibition of bovine capillary endothelial cell migration by angiostatin produced in a cell-free system ($AS_{CF}$). Endothelial cell migration in a Boyden chamber toward a range of concentrations of angiostatin was measured in the presence (solid symbol) or absence (open symbol) of stimulatory bFGF. As a control, single points using elastase-generated angiostatin ($AS_{Elastase}$) are shown (squares). These data demonstrate that $AS_{CF}$ inhibits bFGF-induced endothelial cell migration in a dose-dependent manner, with an $ED_{50}$ of 0.33 μg/ml.

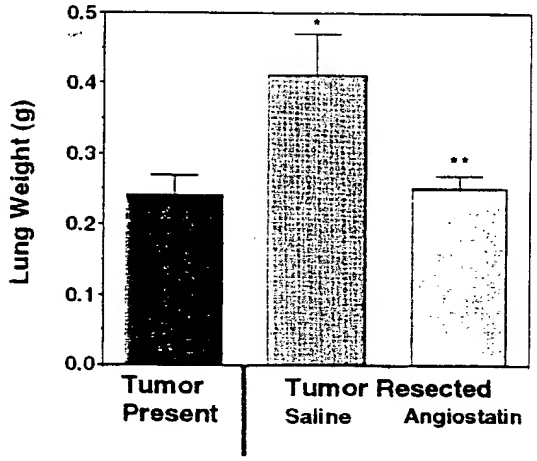


FIG. 4. Angiostatin produced in a cell-free system suppresses the growth Lewis lung carcinoma lung metastases after resection of the primary tumor. The presence of the primary subcutaneous Lewis lung tumor suppressed the expansion of lung metastases (tumor present control). By contrast resection of the Lewis lung tumor and administration of saline resulted in a significant increase in the mean lung mass compared with the tumor present control, confirming primary tumor-mediated suppression of metastatic tumor growth (*, $P < 0.01$). Subcutaneous administration of angiostatin after removal of the primary tumor, significantly suppressed the expansion of lung metastases to levels comparable to the tumor control group (angiostatin compared with saline; **, $P < 0.01$).

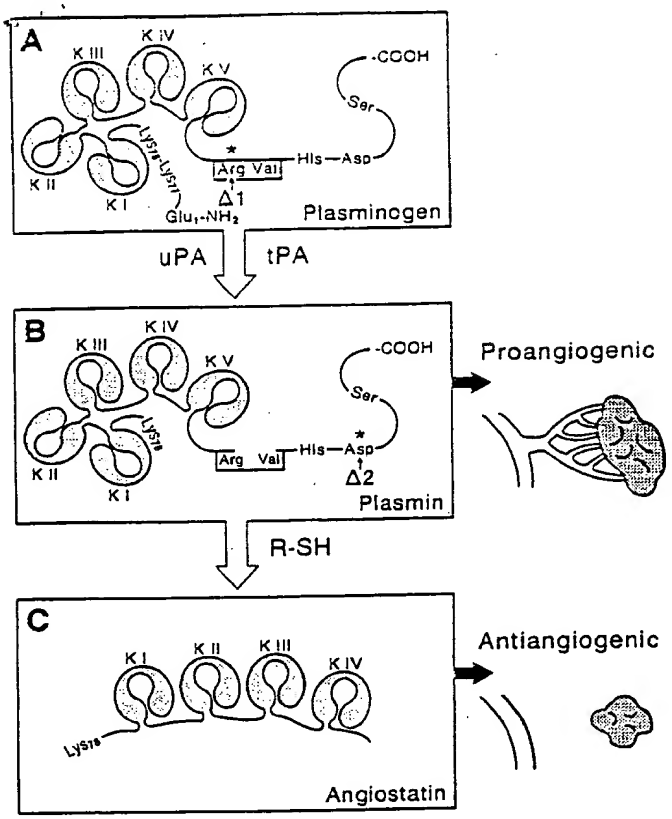


FIG. 5. Conversion of the proangiogenic proteinase plasmin to the angiogenesis inhibitor angiostatin. (*A*) The zymogen plasminogen is converted to the active proteinase plasmin by cleavage of the $Arg_{560}$-$Val_{561}$ peptide bond by plasminogen activators such as uPA and tPA. (*B*) Plasmin is a proangiogenic proteinase capable of degrading a variety of extracellular matrix proteins, facilitating endothelial cell migration and angiogenesis. (*C*) Plasmin in the presence of a FSD is converted to the angiogenesis inhibitor angiostatin. The plasminogen activation site mutant *R561A*, indicated by Δ1, is not cleaved by plasminogen activators, preventing conversion of plasminogen to the plasmin intermediate required for angiostatin generation. The plasminogen mutant *D646E*, indicated by Δ2, is cleaved by plasminogen activators, but the resulting two-chain plasmin is inactive due to the substitution of a catalytically essential aspartic acid residue in the serine proteinase catalytic triad. In the presence of a FSD, the inactive *D646E* mutant plasmin is not converted to angiostatin, demonstrating the requirement for plasmin catalytic activity.

fibrinolysis and the hypercoagulable state often observed in patients with cancer (26).

The angiostatin-generating activity released by human prostate carcinoma cells was not blocked by inhibitors of elastase or metal-dependent proteinases (9). These data suggest a direct mechanism of angiostatin generation by human prostate cancer cells, in contrast to the indirect mechanism of angiostatin generation, dependent upon expression of metalloelastase by tumor-infiltrating macrophages (15). Thus, these data demonstrate alternative models of angiostatin generation, suggesting there may be multiple pathways for the generation of angiostatin.

The identification of a direct mechanism of human prostate cancer-mediated angiostatin generation and the recapitulation of this process in a cell-free system allow for the efficient large-scale production of angiostatin that is antiangiogenic and capable of suppressing the growth of Lewis lung carcinoma metastases. The ability to produce angiostatin in a cell-free system will allow for large-scale production of this protein for *in vivo* testing as an novel anticancer agent. In addition, the elucidation of the components required for plasminogen conversion to angiostatin could permit the direct *in vivo* generation of angiostatin in the patient by administration of a plasminogen activator with a pharmacologic FSD.

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